Population genetics of the *Sporothrix splendens* complex from *Protea* L. in South Africa

by

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Nombuso P. Ngubane March 2017

Abstract

Ophiostomatoid fungi consist of a remarkable assemblage of species that are distantly related (Orders Microascales and Ophiostomatales) and that are grouped based on convergent evolution towards arthropod dispersal. Most of these fungi are known as pathogens of trees. An unusual assemblage of species belonging in the genera *Knoxdaviesia* (Microscales) and *Sporothrix* (Ophiostomatales) were found within infructescences of *Protea* species. Thus far three and nine species, respectively have been described from this niche. Although distantly related, these fungi share the same hosts and vectors with some of them more exclusive in their host selection than others. The reasons for preference towards certain hosts and the different levels of exclusivity are unknown, especially in *Sporothrix*. Also, in contrast to *Knoxdaviesia*, nothing is known regarding their population genetic structure.

In this study we aimed to gain insight into the population genetics of *Sporothrix* species in the *Sporothrix splendens* clade. This was done by investigating the population structure of *S. africana*, *S. protearum* and *S. splendens*. In the second chapter the population structure of *S. splendens* across its entire known distribution range was investigated and compared to its distant relative *K. proteae*. These fungi share the same host (*Protea repens*) and vectors (mites and beetles). In the third chapter the aim was to expand on knowledge of the dispersal of *Protea*-associated ophiostomatoid fungi to include species found in *Protea* species occurring outside the Core Cape Subregion (CCR). To this end, the population structure of two sister species, *S. africana* and *S. protearum*, were investigated in order to ascertain whether gene flow is restricted based on geography or host identity and whether these two similar taxa represented two discrete species. Population genetic structure was assessed using a fast evolving anonymous marker (m128) and the slower evolving beta-tubulin marker. Genetic diversity (haplotype and nucleotide diversity), population differentiation, rates of migration, isolation by distance and the relationship between haplotypes were calculated for both markers. In the case of *S. africana* and *S. protearum*, analyses were partitioned to test the effect of geography and that of host identity. The population structure of *S. africana*, *S. protearum* and *S. splendens* were not structured according to geography and, in the case of the former two, neither were they structured according to host identity. These patterns matched those of *Knoxdaviesia proteae* and *K. capensis*. Also, much like the *Knoxdavesia* species from *Protea*, the *Sporothrix* species showed high genetic diversity, rates of gene flow and no signal for isolation by distance. In addition, two new species (*Sporothrix smangaliso* and *S. nsini*) were discovered in the course of field work and these are described in the forth chapter based on morphology and phylogenetic analyses of data from the ITS, beta-tubulin and calmodulin DNA markers.

Results of this study provide strong evidence of convergent evolution between two distantly related fungal genera driven by adaptation to insect dispersal. In addition, they highlight the importance of the role played by long distance dispersers in shaping populations of *Sporothrix* and *Knoxdaviesia* within *Protea*. The discovery of two additional *Sporothrix* species belonging to two distantly related clades in the genus supports the hypothesis that the *Protea* niche was independently colonised more than once by members of this genus and that some clades have since experienced radiation such as the *S. splendens* clade. However, this radiation does not seem to be driven by host relationships or geography, at least for the non-CCR taxa, and warrants further investigation.

Opsomming

Ophiostomatoid fungi bestaan uit ‘n merkwaardige versameling van spesies wat verlangs verwant is (Ordes Microascales en Ophiostomatales) wat saamgegroepeer is gebaseer op konvergente evolusie vir artropode verspreiding. Meeste van hierdie fungi is bekende patogene van bome. ‘n Ongewone versameling spesies wat behoort aan die genera *Knoxdaviesia* (Microscales) en *Sporothrix* (Ophiostomatales) is in die saadkoppe van *Protea* species gevind. Sover is onderskeidelik vier en nege spesies vanaf hierdie nis beskryf. Alhoewel verlangs verwant, deel hierdie fungi dieselfde gashere en vektore, met sommiges meer eksklusief in hul gasheer seleksie as ander. Die redes vir die voorkeure vir sekere gashere en die verskillende vlakke van eksklusiwiteit is onbekend, veral in *Sporothrix*. Ook, in kontras met *Knoxdaviesia*, is niks bekend oor hulle populasie genetiese struktuur nie.

In hierdie studie het ons gemik om insig te verkry in die populasie genetika van *Sporothrix* spesies in die grootste *Protea*-geassosieerde klade, die *Sporothrix splendens* klade. Dis is gedoen deur die populasie genetiese struktuur van *S. africana*, *S. protearum* en *S. splendens* te ondersoek. In die eerste hoofstuk het ek die populasie struktuur van *S. splendens* oor die hele bekende verspreidingsgebied bepaal, en dit vergelyk met dié van sy verlangs verwante *K. proteae*. Hierdie fungi deel dieselfde gasheer (*Protea repens*) en vektore (meite, kewers en dalk voëls). In die tweede hoofstuk (Hoofstuk 3) het ek gemik om uit te brei op die kennis oor die verspreiding van *Protea*-geassosieerde ophiostomatoid fungi deur spesies wat in *Protea* spesies wat buite die Kaapse Hoof Streek (KHS) voorkom te ondersoek. Om dit te doen is die populasie struktuur van twee susterspesies, *S. africana* en *S. protearum*, ondersoek om te bepaal of geenvloei beperk is gebaseer op geografie of gasheer identiteit en of hierdie twee eenderse taksa twee diskrete spesies verteenwoordig. Populasie genetiese struktuur is bepaal deur gebruik te maak van ‘n vinnig evoluerende anonieme merker (m128) en die stadiger evoluerende beta-tubulin merker. Genetiese diversiteit (haplotipe en nukleotiede diversiteit, populasie differensiasie, en tempos van migrasie, isolasie deur afstand en die verwantskap tussen haplotipes is bereken vir beide merkers. In die geval van *S. africana* en *S. protearum*, is analises gekomparementaliseer om die effek van geografie en gasheer digtheid te toets. Die populasie struktuur van *S. africana*, *S. protearum* en *S. splendens* is nie gestruktureer volgens geografie nie, en in die geval van die eerste twee, ook nie volgens gasheer identiteit nie. Hierdie patrone het ooreengestem met dié van *Knoxdaviesia proteae* en *K. capensisis*. Ook, baie soos die *Knoxdavesia* spesies vanaf *Protea*, het die *Sporothrix* spesies hoë genetiese diversiteit, hoë tempos van geenvloei en geen sein vir isolasie deur afstand geopenbaar nie. Verder is twee nuwe spesies (*Sporothrix smangaliso* en *S. nsini*) ontdek in die verloop van veldwerk, en ek beskryf hierdie twee in die derde data hoofstuk (Hoofstuk 4) gebaseer op morfologie en filogenetiese analises van data van die ITS, beta-tubulin en calmodulin DNS merkers.

Resultate van hierdie studie verskaf sterk bewys van konvergente evolusie tussen twee verlangs verwante fungus genera gedryf deur aanpassing by insek verspreiding. Verder beklemtoon die resultate die belang van die rol wat langafstand verspreiders speel in die vorming van populasies van *Sporothrix* en *Knoxdaviesia* binne *Protea*. Die ontdekking van twee addisionele *Sporothrix* spesies wat aan twee verlangs verwante klades in die genus behoort ondersteun die hipotese dat die *Protea* nis onafhanklik meer as een keer gekoloniseer is deur lede van hierdie genus en dat sommige klades sederdien radiasie ondervind het, soos byvoorbeeld die *S. splendens* klade. Hierdie radiase blyk egter om nie aangedryf te word deur gasheer verwantskappe of geografie nie, ten minste nie vir die ekstra-KHS nie, en regverdig verdere ondersoek.

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GENERAL LITERATURE REVIEW

1.1. Introduction

1.1.1. Taxonomic complexity and the ophiostomatoid fungi

Historically, fungi with long perithecial necks through which sticky spores are pushed to assemble as sticky droplets at their tips were all assigned to *Ceratocystis* Ellis & Halst. (established in 1890) (Upadhyay 1981, Malloch & Blackwell 1993). As more species were described, it became clear that this group was non-monophyletic (Seifert *et al.* 2013). The description of genus *Ophiostoma* H. & P. Sydow in 1919 aggravated the confusion in defining the boundaries of *Ceratocystis*, with ascospores initially confused with conidia (de Beer *et al.* 2014). Eventually the informal, non-monophyletic group, the ophiostomatoid fungi, was recognized to accommodate all fungi characterised by long perithecial necks, hyaline, mucilaginous ascospores and evanescent asci (Wingfield *et al.* 1993). Amongst many others, this group also accommodated species in *Ceratocystis* and *Ophiostoma* (Wingfield *et al.* 1993). The morphological similarities between ophiostomatoid fungi are attributed to convergent evolution driven by adaptation to insect dispersal (Wingfield *et al.* 1993).

Today, the ophiostomatoid fungi accommodates all families in the order Ophiostomatales (de Beer *et al*. 2013a) and some from the order Microascales (De Beer *et al*. 2013b). Historically taxa in these orders were classified in genera based on their asexual or sexual life stages (Hibbett & Taylor 2013), but this has been replaced by the “one fungus, one name” rule through which dual nomenclature has been abolished (Taylor 2011). Names established under the dual nomenclature system have now become synonymised (Hawksworth 2012). Ophiostomatoid fungal genera in the Microascales include *Ceratocystis*, *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas, *Graphium* Corda, *Sphaeronaemella* Helvellae, *Cornuvesica* C.D. Viljoen, M.J. Wingf. & K. Jacobs and *Custingophora* Stolk, Hennebert & Kolopotek (de Beer *et al*. 2013a). Ophiostomatoid fungi in the Ophiostomatales include ten genera; namely, *Ophiostoma*, *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Grosmannia* Goid (de Beer *et al.* 2013b) and *Sporothrix* (Hektoen & C.F. Perkins) Z.W. de Beer, T.A. Duong & M.J. Wingf. (de Beer *et al.* 2016). Many genera that have now been synonymised under these were once considered to constitute separate genera based on their asexual states. Examples of such genera include *Pesotum* Crane & Schoknecht., *Leptographium* Lagerb. & Melin and *Hyalorhinocladiella* Upadh. & Kendr. (de Beer *et al.* 2013b). Until recently *Ophiostoma* and *Grosmannia* were also considered to be synonymous, but Zipfel *et al.* (2006) recognized *Grosmannia* as a distinct genus. *Ophiostoma* is morphologically easily distinguishable

from *Ceratocystiopsis* based on falcate ascospores, with ascospore sheaths and short perithecial necks (Kim 2010).

The demarcation of genus the *Ophiostoma* has been the cause of much confusion. The first revision separated it from *Ceratosystis* (Hausner *et al.* 1993). Later, *Ophiostoma* was further subdivided into *Ophiostoma sensu stricto* and *Ophiostoma sensu lato* (de Beer *et al.* 2013a). De Beer *et al*. (2013a) proposed that *Ophiostoma s.l.* represented a distinct genus. Subsequently, all species that resolve in *Ophiostoma s.l.* have been accommodated in *Sporothrix* and this genus has been redefined accordingly (de Beer *et al.* 2016). The revision of this genus was done such that important tree pathogens such as *O. ulmi* (Buisman) Nannf. (and its respective subspecies) and major causal agents of sporotrichosis (such as *S. schenckii* Hektoen & C.F. Perkins) could retain their names to minimise confusion (de Beer *et al.*2016).

1.1.2. Pathogenic ophiostomatoid fungi

Many ophiostomatoid fungi are aggressive and important tree pathogens (Wingfield *et al.* 1993, Jacobs & Wingfield 2001). These are mostly found in association with bark beetles that primarily infest conifers in the Northern Hemisphere (Perry 1991). *Ceratocystis manginecans* M. van Wyk, Al Adawi & M.J. Wingf., for example, causes a serious wilt disease in mango trees (*Mangifera indica* L.) in Oman and Pakistan (Al Adawi *et al*. 2014). This fungus is usually found in association with the wood boring beetle, *Hypocryphalus mangiferae* (Coleoptera: Scolytinae) (Al Adawi *et al*. 2006). Together these organisms have killed thousands of mango trees, resulting in substantial economic losses (Van Wyk *et al*. 2007).

There have been numerous reports of Microascalean ophiostomatoid fungi from hosts that are both exotic and native to southern Africa (Morris *et al*. 1993, Van der Linde *et al*. 2012, de Beer *et al.* 2014). These include *Ceratocystis albifundus* Ellis & Halsted, which causes serious wilting and gummosis in *Acacia mearnsii* De Wild. trees in South Africa (Morris *et al*. 1993), Kenya (Roux *et al.* 2005), Tanzania (Roux *et al*. 2005) and Uganda (Roux *et al*. 2001). This fungus has also been isolated from a number of native South African hosts including *Senegalia caffra* (Thunb.) P.J.H. Hurter & Mabb., *Burkea africana* Hook, *Combretum molle* R.Br. ex G. Don, *Faurea saligna* Harv, *Ozoroa paniculosa* (Sond.) R. Fern. & A. Fern., *Terminalia sericea* Cambess (Roux *et al*. 2007) and *Protea* species (Gorter 1977, Roux *et al*. 2004, 2007). A number of studies suggested that this is a native African fungus (Roux *et al.* 2001, 2007). Pathogenicity tests showed that another native fungus, *Ceratocystis tsitsikammensis* Kamgan & Jol. Roux, infects *Senegalia nigrescens* Oliv., *Sclerocarya birrea* (A. Rich.) Hochst., *Rapanea melanophloeos* (L.) Mez (Kamgan *et al*. 2008) and *Virgilia* Poir. trees (Van der Colff *et al.* 2016). *Graphium adansoniae* Cruywagen, Z.W. de Beer & Jol. Roux was identified as a pathogen on baobab trees (*Adansonia digitata* L.) in Madagascar and South Africa (Cruywagen *et al.* 2010).

Globally, numerous Ophiostomatalean fungi have been responsible for substantial economic losses. For example, the insect–fungal association between *Grosmannia clavigera* (R.C. Rob. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. and the mountain pine beetle has been responsible for the destruction of over 16 million hectares of lodge pole pine forests in Canada (Tsui *et al.* 2012). *Ophiostoma ulmi*, *O. novo-ulmi* Brasier and *O. ips* (Rumb.) Nannf. are among the best researched *Ophiostoma* species owing to their devastating impacts to forestry industries in the Northern Hemisphere (Hintz 1999, Paoletti *et al.* 2006, Solla *et al.* 2008, Brasier & Kirk 2010, Plichta *et al.* 2016). *Ophiostoma ulmi* and *O. novo-ulmi* cause Dutch elm disease, a vascular wilt disease, which has devastated indigenous elm trees across most of Europe and North America (Sinclair & Lyon 2005). *Ophiostoma ips* is a sapstain causing fungus that has caused major losses to forestry industries (Seifert 1993).

Ophiostomatalean pathogens have also been found infecting native and non-native hosts in South Africa. Specific examples include *Ophiostoma piliferum* (Fr.) Syd. & P. Syd., *O. floccosum* Math.-Käärik, *S. stenoceras* (Robak) Nannf and *S. abietina* (Marm. & Butin) Z.W. de Beer, T.A. Duong & M.J. Wingf. (Zhou *et al.* 2001, Kamgan *et al*. 2008). All of these fungi are found in association with non-native hosts and are carried by pine infesting bark beetles *Hylastes angustatus* Herbst, *Hylurgus ligniperda* Fabricus and *Orthotomicus erosus* Wollaston (Zhou *et al.* 2001). *Ophiostoma quercus* (Georgévitch) Nannf., a globally common fungus, has also been recorded from some non-native hosts in South Africa, including *Eucalyptus grandis* W. Hill (De Beer *et al.* 1995). A *Ophiostoma pluriannulatum*-like fungus was isolated from several hosts, including *A. mearnsii* and *R. melanophloeos*, and was shown to be a pathogen in both cases (Musvuugwa *et al*. 2016b).

The order Ophiostomatales is also known for its assemblage of species that are human and animal pathogens. The most notable of these pathogens are found in *Sporothrix*, specifically the *Sporothrix schenckii* (type species of *Sporothrix*) complex (Kano *et al.* 2015; Rodrigues *et al.* 2015). They consist of an assemblage of closely related species that cause sporotrichosis in humans and animals such as cats (Rodrigues *et al.* 2013, 2014b, Teixeira *et al.* 2015). Species in this complex include *S*. *mexicana* Marimon, Gené, Cano & Guarro, *S. pallida* (Tubaki) Matsush., *S. globosa* Marimon, Gené, Cano, *S. brasiliensis* Gené, Cano & Guarro and *S. luriei* (de Beer *et al.* 2016). Numerous cases of sporotrichosis due to *S. schenckii* have been recorded in South Africa (Vismer & Hull 1997).

1.1.3. Symbiotic interactions

Ophiostomatoid fungi are entomochoric, meaning they are morphologically adapted for arthropod spore dispersal (Wingfield *et al.* 1993, Six & Paine 1997). Many wood- and sap-feeding beetles vector ophiostomatoid fungi, especially species in the genera *Ceratocystis*, *Ceratosystiopsis*, *Grosmannia*, *Leptographium*, *Ophiostoma* and *Sporothrix* (Upadhyay 1981, Wingfield *et al*. 1993,

Jacobs & Wingfield 2001, Zipfel *et al*. 2006, Musvuugwa *et al*. 2015). Sticky spores produced by these fungi are pushed to the tips of conidiophores or elongated ascomatal necks (Upadhyay 1981, Malloch & Blackwell 1993). Aggregated as sticky masses in this position, they easily come into contact with beetles that transport them to their galleries and pupal chambers or to wounds on trees (Zhou *et al.* 2007a). Some beetles have specialised structures for fungal transport, suggestive of a mutualistic relationship (Six & Paine 1998, Klepzig *et al*. 2001).

Pathogenic ophiostomatoid fungi like *Ceratocystis* spp. often need wounded host plants in order to inoculate and cause infections (Kile 1993). Fresh wounds attract spore-carrying insects that feed on sap (Barnes *et al.* 2003). In this way fungal spores are dispersed by the movement of these insects between wounds, thus carrying the ascospores from diseased to uninfected hosts (Upadhyay 1981). The beetle, *Dendroctonus frontalis*, has a mutualistic association with *Ceratosystiopsis ranaculosus* (Klepzig *et al.* 2001). This beetle keeps pure cultures of *C. ranaculosus* within mycangia, from where they may deposit them into the area around the eggs deposited into the wounds they cause on the trees (Barras & Taylor 1973). In turn, these fungi serve as source of nourishment to the larval stages of the beetle (Klepzig *et al.* 2001, Klepzig & Six 2004). The association between the beetles and ophiostomatoid fungi, therefore, seems to be mutualistic (Klepzig *et al.* 2001, Kolařík & Hulcr 2009). In South Africa, numerous *Sporothrix* species have been found in association with Scolytinae and Platypodinae beetles including *Sporothrix aemulophila* (Musvuugwa *et al.* 2015), but the nature of these interactions have not yet been studied.

In some cases, mites that are phoretic on beetles serve as primary vectors of ophiostomatoid fungi, which demotes the beetles to secondary spore vectors (Aghayeva *et al*. 2004, Zhou *et al.* 2007b, Roets *et al*. 2011, Tsui *et al*. 2012). Mites also often have a mutualistic relationship with the ophiostomatoid fungi they carry (Moser 1985, Bridges & Moser 1983). Much like the mycangia present on the beetles, some mites have specialised structures, sporothecae, in which spores are assembled and then dispersed (Roets *et al*. 2007). Spores of ophiostomatoid fungi such as *Ophiostoma minus* (Hedgc.) H and I' Sidow have been isolated from mites like *Tarsonemus ips* Lindquist, *T. krantzii* Smiley & Moser and *T. furasii* Cooreman (Acarina: Tarsonemidae) (Moser 1985, Bridges & Moser 1983). Ophiostomatoid fungi can also serve as a source of nutrition to the mites (Klepzig *et al.* 2001, Zhou *et al*. 2007b).

Similar to these Northern Hemisphere examples, studies on the dispersal of comparatively newly discovered *Protea*-associated ophiostomatoid fungi have demonstrated inter-organismal mutualisms (Roets *et al*. 2011). In this system, mites serve as the primary vectors (Roets *et al.* 2007, 2011), while beetles are secondary vectors that transport spore-carrying mites between infructescences (Roets *et al.* 2005, 2006a, 2011). Roets *et al*. (2007) showed that some of these

mite species feed exclusively on *Sporothrix* species, confirming a mutualistic association between them. The beetles that vector spore-carrying mites in the *Protea* system include *Genuchus hottentottus* Fabricius and various *Trichostetha* spp. (Roets *et al.* 2006a, 2007, 2011). These beetle species are also involved in *Protea* pollination (Johnson & Nicolson 2001, Steenhuisen & Johnson 2012a, Steenhuisen *et al.* 2012).

1.1.4. Non-pathogenic ophiostomatoid fungi: The *Protea*-associated species

In addition to the pathogenic ophiostomatoid fungi, a large number of non-pathogenic ophiostomatoid fungi have been documented in some sub-Saharan African countries (for example Marais & Wingfield 1997, 2001, Roets *et al*. 2010). These species belong to the genera *Knoxdaviesia* (Microascales) and *Sporothrix* (Ophiostomatales). They are found in association with native *Protea* species.

The genus *Protea* (Proteaceae) contains approximately 92 species in southern Africa (Rebelo 2001), of which about 69 are restricted to the CCR (Rourke 1980, Manning & Goldblatt 2012). *Protea* species are of importance to biodiversity conservation and their flowers are important to the horticulture industries, both locally and internationally (Coetzee & Littlejohn 2001, Crous *et al*. 2004). Serotinous *Protea* infructescences house a number of arthropods (Lee *et al*. 2005, Roets *et al.* 2007, Theron *et al.* 2011), fungi (Marais & Wingfield 1997, Roets *et al.* 2007, Kamgan *et al.* 2011), bacteria (Human *et al.* 2016) and other taxa, forming a micro-ecosystem. Some important pollinating insects are also encountered here (Coetzee 1989, Wright 1990), some of which are known to play a role in the dispersal of ophiostomatoid fungi (Roets *et al.* 2007, 2009a, 2011).

Various pollination syndromes have been noted within *Protea*, including pollination by vertebrates (rodents and birds) and insects (beetles and honeybees) (Rebelo 2001). Beetle pollination has only recently been confirmed in *Protea* (Steenhuisen & Johnson 2012b), with members of Scarabaeidea (*Protea* beetles and monkey beetles) recorded as the dominant pollinators (Johnson & Nicolson 2001). Steenhuisen and Johnson (2012b) demonstrated that pollination by cetoniid beetles (in addition to birds) are especially prevalent in grassland species (found in the northern and eastern parts of southern Africa) such as *Protea dracomontana* Beard and *Protea caffra* Meisn. Gurney's sugarbird has been recorded frequenting *P. caffra*, which has been identified as one of the major nectar sources for these sugarbirds (Skead 1967, Calf *et al.* 2001). *Protea repens* L., one of the most widespread species in the southern parts of South Africa, is self-compatible (Coetzee & Littlejohn 2007), but both birds and beetles have also been implicated in its pollination (Rebelo 2001).

After pollination and subsequent fertilisation, flower heads of some *Protea* species mature into long-lived and conspicuous infructescences (seed cones) (Bond 1985). The cones confer a fitness advantage as they deter predators by offering protection to seeds (Mustart *et al*. 1995). These stores remain attached to the plant awaiting favourable conditions for seed release and dispersal (Rebelo 2001). This strategy is referred to as serotiny (Midgley & Enright 2000) and is observed in numerous *Protea* species (Rebelo 2001). The plant will store the seeds until the water connection between the plant and cone is severed (Rebelo 1995), which usually occurs when plants are killed by fire. After fire the seeds are dispersed across the landscape and germinate after the first winter rains (Bond 1984, Rebelo 2001). Serotiny allows for the accumulation of a large seed bank and release when granivory is low. In addition, chances of seedling establishment are high due to decreased post-fire competition (Midgley & Enright 2000).

The infructescences and inflorescences of *Protea* species also house different mite species (Roets *et al.* 2008, 2011, Theron *et al.* 2011), *e.g. Proctolaelaps vandenbergi* (Rebelo 2001, Fleming & Nicolson 2003, Theron *et al.* 2011). These mites feed on nectar (Coetzee & Giliomee 1985) and are phoretic on birds and beetles (Theron 2011). Some of these mites have been demonstrated to play a role in the dispersal of *Protea*-associated ophiostomatoid fungi (Roets *et al.* 2006a, 2007, 2011), and their potential role in *Protea* pollination is currently being investigated (Theron-de Bruin *et al. pers. comm*.).

In the *Protea* system, only a few insects are known to carry *Sporothrix* (Roets *et al.* 2006c). However, various mites (*Proctolaelaps vandenbergi* Ryke, two species of *Tarsonemus* Canestrini and Fonzago, and one *Trichouropoda* Berlese species) frequently carry *Sporothrix* propagules (Roets *et al*. 2007). Species confirmed to be present on these mites include *S. splendens* G.J. Marais & M.J. Wingf., *S. palmiculminata* (Roets, Z.W. de Beer & MJ Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *S. phasma* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. (Roets *et al.* 2007). In addition to *Protea*-associated *Sporothrix* species, isolations revealed that Acari species in the genus *Trichouropoda* are the most common and likely vectors of *Knoxdaviesia proteae* M.J. Wingf., P.S. van Wyk & Marasas (Roets *et al*. 2011). Further experiments demonstrated that these mites are also able to carry viable *Sporothrix* spores whilst phoretic on *Genuchus hottentottus* beetles and that these mites can transfer *K. proteae* spores to uncolonised substrates *in vitro* (Roets *et al.* 2011). What made this even more intriguing was that the vectors of *Sporothrix* species associated with *Protea repens* belong in the same genus as species implicated in the dispersal of *Ophiostoma* species from the Northern Hemisphere (Mercado *et al*. 2014).

The concept of host association, host specificity and exclusivity are of great importance in understanding fungal biology and estimating fungal diversity. While often used interchangeably,

host exclusivity and specificity have been defined by Zhou & Hyde (2001) as distinct and different concepts with host exclusivity referring to a strictly saprobic fungus that exclusively occurs on a specific host or a on a restricted range of related host plants while host specificity only applies to a strict relationship between a fungus and a live plant. Ophiostomatoid fungi found in association with *Protea* are considered as saprobes as they are either found in association with dead floral parts or with soils. Different ophiostomatoid fungi show different levels of host exclusivity with their *Protea* hosts. However, the reasons for host selection and these different levels of exclusivity are not well understood. Roets *et al.* (2012) demonstrated that while temperature and relative humidity affected growth of ophiostomatoid fungi in *Protea*, they did not explain the propensity of some fungi to be host exclusive, while others are less particular. *Protea*-associated *Knoxdaviesia* and *Sporothrix* species are distant relatives belonging the Phylum Ascomycota under the orders Microascales and Ophiostomatales, respectively (Wingfield *et al.* 1999, de Beer *et al.* 2013b, Roets *et al.* 2013). They, therefore, appear to have independently evolved associations with *Protea* (Wingfield *et al.* 1999, Roets *et al.* 2009, 2013a). However, *Protea* infructescences offer an ideal, moist environment within which the ophiostomatoid fungi can thrive and, when present, they have been found to be the most prevalent fungi in *Protea* infructescences (Roets *et al.* 2005). This suggests that they may exclude other, more detrimental, fungi from these seed carrying structures, thereby offering some protection to their *Protea* hosts (Lee *et al.* 2005). This needs detailed further study.

Nine *Knoxdaviesia* species have been found in Thailand (Pinnoi *et al*. 2003), Costa Rica (Kolařík & Hulcr 2009) and South Africa (de Beer *et al.* 2013a). *Knoxdaviesia capensis*, *K. proteae*, *K. serotectus* (van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf., *K. ubusi* (Van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf., *K. wingfieldii* (Roets & Dreyer) Z.W. de Beer & M.J. Wingf. and *K. suidafrikana* (Morgan-Jones & R.C. Sinclair) Z.W. de Beer & M.J. Wingf. are indigenous to South Africa (de Beer *et al.* 2014). Only three of the known South African species have colonised the *Protea* infructescence niche. *Knoxdaviesia proteae* is restricted to *P. repens* (Marais & Wingfield 2001) and *K. wingfieldii* has been described from *P. caffra* (Crous *et al*. 2012). *Knoxdaviesia capensis* has a much wider host range that includes *P. repens* (although rarely encountered here; Aylward *et al.* 2015), *P. longifolia* Andrews, *P. neriifolia* R. Br., *P. lepidocarpodendron* L., *P. coronata* Lam. and *P. laurifolia* Thunb. (Marais & Wingfield 2001).

Species of *Sporothrix* associated with *Protea* infructescences are morphologically similar and phylogenetically closely related to Northern Hemisphere taxa, known to be insect vectored (Marais & Wingfield 1997, Wingfield *et al.* 1999). Currently, all *Protea*-associated *Sporothrix* species are known only from South Africa (Marais & Wingfield 1997, 2001, Roets *et al.* 2006a) and Zambia (Roets *et al*. 2010). To date, nine *Sporothrix* species have been described from *Protea* infructescences; namely *Sporothrix africana* G.J. Marais & M.J. Wingf., *S. gemella* (Roets, Z.W. de

Beer & P.W. Crous.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. palmiculminata*, *S. phasma*, *S. protearum* Marais and M.J. Wingfield, *S. protea-sedis (*Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf, *S. splendens*, *S. variecibatus* Roets, Z.W. de Beer & P.W. Crous and *S. zambiensis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf (Marais & Wingfield 1994, 1997, 2001, Roets *et al.* 2006a, 2008). At least one undescribed species is also known from this niche (Roets *et al*. 2013).

The apparent host exclusivity of *Knoxdaviesia* and *Sporothrix* species associated with *P. repens* may be ascribed to the phylogenetic uniqueness of this host in relation to all other ophiostomatoid-associated *Protea* species (Barraclough & Reeves 2005, Valente *et al*. 2010), coupled with the uniqueness of this hosts' chemistry (Roets *et al.* 2012). However, the recent discovery of the generalist *Knoxdaviesia capensis*, although in low abundance, in *Protea repens* (Aylward et al. 2015a) exacerbates the mystery surrounding the host selection of *Protea*-associated ophiostomatoid fungi. Furthermore, the recent discovery of *S. splendens* in *P. neriifolia* (Theron-de Bruin *et al. pers. comm.*) highlights the fact that plenty still remains unknown about the biology and ecology of these ophiostomatoid fungi. As in *Knoxdaviesia* associated with *Protea* hosts, differential levels of host exclusivity have been observed across *Protea*-associated *Sporothrix* spp. (Roets *et al*. 2013). It is also quite possible that the species that have only been encountered in one particular host thus far also occur in other hosts, but have not been collected yet.

Outside the CCR, *Sporothrix* species are found in three *Protea* species; namely, *Protea caffra*, *P. dracomontana* and *P. gaguedi* J.F. Gmel (Marais & Wingfield, 1997, 2001, Roets *et al.* 2008, 2010). *Sporothrix africana*, originally thought to be specific to its *Protea gaguedi* host (Marais & Wingfield 2001), has also been isolated from *P. dracomontana* and *P. caffra (Roets et al.* 2006a). *Protea caffra* also hosts *Sporothrix gemella*, Z.W. de Beer & P.W. Crous (Roets *et al*. 2009b) and *Sporothrix protearum* (Marais & Wingfield 1997), both of which are thought to be exclusive to this host (Marais & Wingfield 1997, Roets *et al.* 2009b). *Sporothrix protea-sedis* and *S. zambiensis*, found outside of South Africa, are also known exclusively from *P. caffra* (Roets *et al.* 2010).

Sporothrix species found within the CCR are better studied and have been found in comparatively more *Protea* species than their non-CCR relatives (Marais & Wingfield 1994, Roets *et al.* 2006a). The fact that the CCR has a higher diversity and abundance of serotinous *Protea* species (Rebelo 2001) possibly contributes to this. Considering the abundance and rich diversity of serotinous *Protea* species in the CCR, it stands to reason that many more species of ophiostomatoid fungi remain undiscovered. *Sporothrix phasma* is found across a wide range of *Protea* hosts including *P. laurifolia, P. lepidocarpodendron, P. longifolia* and *P. neriifolia* (Roets *et al*. 2009b). In contrast, *Sporothrix palmiculminata* and *S. splendens* have only been found on *Protea repens* (Roets *et al*. 2006a). Previous reports of *S. splendens* on other *Protea* hosts (*Protea coronata*, *P.*

lepidocarpodendron, *P. neriifolia*) in the Western Cape have subsequently been shown to, in fact, be *S. phasma* (Roets *et al*. 2009b). However, Theron-de Bruin *et al.* (*pers. comm*.) has recently collected a few isolates of *S. splendens* from *P. neriifolia* raising the possibility that, much like *K. capensis*, it can survive on less preferred hosts, at least in the very early flowering stages, but in lower numbers than it does on *P. repens*.

Based on two molecular markers (internal transcriber spacer (ITS) and β-tubulin), the *Protea*-associated *Sporothrix* species form several distinct clades within the genus (Roets *et al*. 2013), including:

- A clade containing *Sporothrix protea-sedis*, *S. gemella* and *S. palmiculminata* (the *S. gemella* clade)
- *Sporothrix phasma* that is distantly related to all other species associated with *Protea*
- *Sporothrix variecibatus* that groups with both South African and non-South African *Sporothrix* species. This clade consists of members from conifers (*S. abietina* Marm. & Butin) (De Beer *et al.* 2003), conifer infesting beetles (*S. aurorae* (X.D. Zhou & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf.) and deciduous trees (*S. lunata* (Aghayeva & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *S. fusiformis* (Aghayeva & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf.) (De Meyer *et al*. 2008)
- A clade containing the majority of species, including *Sporothrix splendens*, *S. zambiensis*, *S. africana*, *S. protearum* and one undescribed species from *P. caffra* (the *S. splendens* clade)

1.1.4.1. The *Sporothrix splendens* clade

The polyphyletic origin of *Protea*-associated *Sporothrix* species proposes multiple independent invasions of the *Protea* host by *Sporothrix* species followed by speciation events in certain lineages (Roets *et al.* 2013). Such clades include the *Sporothrix splendens* clade, which consists of morphologically similar species (Roets *et al.* 2010). Although nearly identical in morphology and based on molecular data, *S. protearum* and *S. africana* are sister species (Zipfel *et al*. 2006) sharing the same *P. caffra* host (Marais & Wingfield 1997, Roets *et al.* 2006a). *Sporothrix africana* has a wide geographical range (and occurs in many *Protea* species), which may stretch from Mpumalanga Province to KwaZulu-Natal Province (Roets *et al.* 2006a). *Sporothrix protearum* is thus far only known from a single host (*P. caffra*) (Marais & Wingfield 2001), but this is a widespread host with a distribution that extends from the Eastern Cape Province (South Africa) to Ethiopia (Rebelo 2001). Recent collections from Gauteng Province and KwaZulu-Natal Province have revealed that there are isolates that cannot be definitively placed as either *S. protearum* or *S.*

africana (based on molecular data). This brings into question the validity of recognizing them as separate species (Roets, unpublished data).

Sporothrix splendens groups as sister to *S. africana* and *S. protearum* (De Meyer *et al.* 2008, Roets *et al.* 2009b). It is predominantly found within *P. repens*, a host with a distribution that does not overlap with the hosts of *S. protearum* and *S. africana*. The pollinators (and therefore the fungal vectors) of this *Protea* species are very different to those of *P. gaguedi* and *P. caffra*, with the former relying on bird (such as the Cape sugarbird) and insect (such as the *Protea* beetle) pollination (Rebelo 2001) and the latter two species mostly relying on beetles (Steenhuisen & Johnson 2012b). The population structure of *S. splendens*, which also has a very wide distribution, may therefore be very different to that of *S. africana* and *S. protearum*. *Sporothrix zambiensis* (an associate of *P. caffra* from Zambia) and an undescribed taxon from *P. caffra* in Gauteng are sister species to the above mentioned members of the *S. splendens* clade (Roets *et al*. 2013). These mixed levels of host exclusivity indicate that the species/taxon boundaries and the factors that determine and/or maintain these boundaries are unclear in this clade, and require focussed research attention.

In addition to taxonomic uncertainties of some of the fungi in the *S. splendens* clade, the identification of the associated hosts is difficult, as *P. caffra* resembles *P. gaguedi* and *P. dracomontana* in areas where they overlap (Rebelo 2001, Roets *pers. comm*). When in sympatry *P. caffra* is known to hybridise with either *P. gaguedi* or *P. dracomontana* (Rebelo 2001), thus the presence of potential hybrids further complicates any morphology-based distinctions. In addition, the pollinators (and presumably then also the vectors of the fungi) are also similar for these host species (Steenhuisen & Johnson 2012b) and further complicates any inferences of host specialisation associated with these two *Sporothrix* species. In attempts to resolve this, fungal collections are critically needed from areas where these hosts co-occur in order to understand the biological complexity of this system. Such collections should help us answer important questions about the geographical and/or host related structure of populations of *S. africana* and *S. protearum* associated with these *Protea* species.

1.2. Study rationale

Population genetic studies have been conducted investigating the dispersal patterns of species in the Ophiostomatales. A microsatellite-based population genetic analysis of *Grosmannia clavigera* (Robinson-Jeffrey & R.W.Davidson) Zipfel, Z.W.de Beer & M.J.Wingf. revealed multiple introductions of this species and its vector into different parts (*e.g.* Fort St. James, Houston) of Canada (Tsui *et al.* 2012). The population analysis of *Grosmannia alacris* M.J. Wingf. and Marasas using both microsatellite and mating type markers revealed high genetic diversity and the presence of a sexual phase, which had never been detected in nature in South Africa (Duong *et al.* 2015).

Interestingly, microsatellite markers revealed inconclusive results for the exact Northern Hemisphere origin of the non-native *Ophiostoma ips* in the Southern Hemisphere, but did suggest that within the native range in the Northern Hemisphere, North America could house the original population (Zhou *et al.* 2007). Investigations into the population diversity of species in the *S. schenckii* complex revealed a dominance of a single allele and a few additional less prevalent alleles (Rodriguez *et al.* 2013). The microsatellites-based population genetics of *Protea*-associated *Knoxdaviesia* species in the CCR consistently revealed that these species have high gene flow resulting in populations with very little genetic differentiation levels (Aylward *et al.* 2014b, 2015, 2017).

The main aim of this study was to understand the dispersal and host exclusivity of the South African members of the *S. splendens* clade. In order to achieve this, individuals from eight populations of *S. splendens*, and 12 populations of *Sporothrix africana* and *S. protearum* were collected from across the South African distribution ranges of the *Protea* species known to host these fungi. This study served a dual role as it not only oadded to the limited population genetics knowledge in the Sporothrix genus but also the limited population genetics knowledge on the *Protea*-associated ophiostomatoid fungi.

***Aim 1 (Chapter 2):* Study the population genetic structure of *Sporothrix splendens* across the geographical range of its *P. repens* host in the CCR.**

This objective was prompted by recent work on the population genetic structure of *Knoxdavesia proteae* on its *Protea repens* host in the CCR (Aylward *et al*. 2014b). Aylward *et al.* (2014a) designed microsatellite markers to study the population genetic structure of *K. proteae.* Aylward *et al*. (2014b) found complete panmixia between distantly located populations (approximately 250 km) of *K. proteae* leading to the hypothesis of bird involvement in long distance dispersal of this fungus. Aylward *et al*. (2015) showed that long distance dispersal plays a critical role in shaping populations of *K. proteae* when recolonising new *Protea* stands after fire.

The objective was to first design microsatellite primers (based on the recently sequenced *S. splendens* genome; CMW23050, *unpublished*) in order to study the population structure of *S. splendens*, *S. africana* and *S. protearum.* However, when microsatellites were designed and tested for *S. splendens* they displayed high levels of variability including in the conserved areas around the microsatellite. This pattern was consistently observed in all 35 tested microsatellites, rendering microsatellites unusable for the purpose of this study. This was very problematic as it lay at the core of the proposed study, we were forced to search for slowere evolving markers.Thus elongation factor, calmodulin, ITS and beta-tubulin markers were tested for suitability in studying populations of *S. africana*, *S. protearum* and *S. splendens*. The calmodulin and elongation factor

markers have been previously used to study populations of species in the *S. schenckii* complex (Rodrigues *et al.* 2013).The available primers for these two markers failed to anneal and amplify the region of interest in the *S. splendens* clade, and the only primer pair that worked (CL3F and CL3R) was difficult to work with as it often failed to amplify. The slow evolution of the ITS region, and therefore low differentiation between closed related taxa, also made this marker unsuitable. Due to its highly variable introns, beta-tubulin showed appropriate levels of variation thus making it an ideal marker for our purposes. In addition to this marker, one of the microsatellite markers were also chosen for population-level analyses as it showed enough variation across individuals. Two custom primers were therefore developed for the marker m128 (so named for the *S. splendens* contig it is located on in the sequenced genome).

Using beta-tubulin and m128 markers, we investigated the genetic diversity, gene flow, population structure and population differentiation of *S. splendens* across the entire geographical range of its *P. repens* host and compared this to what is known for *K. proteae*. It was expected that, due to the ecological similarities between these fungi, *S. splendens* would show near panmictic population structure over a very wide geographic range, and possibly throughout the extended range of the entire *P. repens* host.

Aim 2 (Chapter 3): **The main aim of this study was to build on the meagre ecological knowledge of *Sporothrix* species associated with *Protea* hosts from the northern parts of South Africa.**

Currently, the only population genetic studies on *Protea*-associated ophiostomatoid fungi focus on *Knoxdaviesia proteae* on *P. repens* (Aylward et al. 2014a, 2014b) and *K. capensis* on multiple *Protea* hosts in sympatry (Aylward *et al.* 2017). Both these species are restricted to the CCR. Using microsatellites, Aylward *et al*. (2017) found that *K. capensis* moves freely between hosts regardless of their identity and that geographic distances did not restrict gene flow for either *K. capensis* or *K. proteae* (Aylward et al. 2014a, 2014b). This provided additional evidence of the role birds played in the long range dispersal of ophiostomatoid fungi. Theron-de Bruin *et al.* (*pers. comm*.) confirmed that birds serve as long distance dispersers of *S. splendens* found on the same *P. repens* host and *S. phasma* that dominates some other host plants. The near panmictic population structures of these *Knoxdaviesia* species highlight the effectiveness of the dispersers of these fungi. In Chapter 3, the role of geography and host identity in shaping populations of *S. africana* and *S. protearum* were investigated. This was expected to increase our insights into the biology of *Sporothrix* species on *Protea* hosts across extended geographic areas and in *Protea* species found outside the CCR.

Literature scouring into *Sporothrix africana* and *S. protearum* revealed that there has been limited collection of individuals belonging to these species, restricting our knowledge of their general biology and host relationships. In addition to this, when fungal biology research moved from morphology based identification to molecular-aided tools, the type specimens of these species were not genetically profiled as ex-type cultures were not preserved or, when these could be traced, they were all dead. As a result, representative individuals of these species currently in collections may not correlate with the names they bear. Thus the second objective was to use molecular markers to study these taxa and to attempt to unravel the phylogenetic positioning of populations of individuals believed to belong to *S. protearum* and *S. africana*. In addition, some of the isolates identified in the literature were also included in this study in order to identify their position in relation to our collections.

Aim 3 (Chapter 4): **To describe the two new species discovered in the course of the field work for Chapter 3.**

The objective was to confirm the taxonomic novelty of these taxa through morphological and molecular techniques (using the ITS, beta-tubulin and calmodulin markers). The phylogenetic position of these taxa was validated using Bayesian Inference methods and Maximum Likelihood.

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CONVERGENT EVOLUTION LEADS TO SIMILAR POPULATION GENETIC ATTRIBUTES IN DISTANTLY RELATED *PROTEA* FLOWER-ASSOCIATED FUNGI

1.4. Abstract

Convergent evolution in ophiostomatoid fungi served as basis for establishing this artificial group of arthropod-associated fungi. Closely matching ecologies should result in comparable population genetic profiles of even distantly related species. In this study we investigated whether the population genetic structure of *Sporothrix splendens* (Ophiostomatales) matches that of its distant relative, *Knoxdavesia proteae* (Microascales), both of which grow within the flower heads of *Protea repens* trees and share the same mite, beetle and possibly bird spore-vectors. The population genetic structure of *S. splendens* was assessed using a fast evolving anonymous marker (m128) and the slower evolving beta-tubulin marker across eight populations collected throughout the distribution range of *Protea repens*. Genetic diversity (haplotype and nucleotide diversity), population differentiation, rates of migration, isolation by distance and the relationship between haplotypes (haplotype network) were calculated for both markers and compared to those of *K. proteae* from a previous study. The results revealed a high genetic diversity in *S. splendens*, a mix of near panmixia and great differentiation between populations, high rates of migration and a lack of isolation by distance. These attributes were very similar to those of *K. proteae*, at least when comparing populations from the same geographical areas. These results show that these fungi have similar population genetic attributes most likely driven by their shared host and vectors, and add credence to the hypothesis that ophiostomatoid fungi from this niche may also be dispersed by birds.

Key words: Core Cape Subregion, genetic variation, South Africa, *Ophiostoma, Sporothrix stenoceras-Sporothrix schenckii* complex

1.5. Introduction

The ecology of numerous pathogenic ophiostomatoid fungi (Wingfield *et al*. 1993) has been well-studied globally (De Beer *et al.* 2003, Brasier & Kirk 2010, Rangel-Gamboa *et al.* 2015, Plichta *et al.* 2016), but to date only a few non-pathogenic members have received some attention. One such

example is the work conducted on the unusual members associated with the African endemic plant genus *Protea* L. (Marais & Wingfield 2001, Roets *et al.* 2006a, 2007, 2010, 2011) that belong to the genera *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas (Microascales) and *Sporothrix* (Z.W. de Beer, T.A. Duong & M.J. Wingf) Hektoen & C.F. Perkins (Ophiostomatales) (Wingfield *et al.* 1999, De Beer *et al.* 2013, Roets *et al*. 2013, De Beer *et al*. 2016). They occupy the inflorescences and infructescences of various *Protea* species, especially those that are serotinous (Roets *et al.* 2012). It has been proposed that, when present, these fungi may exclude more detrimental fungi from infructescences, thereby offering some protection to their *Protea* hosts (Lee *et al*. 2005). However, this has not yet been confirmed.

Nine *Sporothrix* species have been described from the infructescences of *Protea* trees (Marais & Wingfield 1997, 2001, Roets *et al.* 2006a). These include: *Sporothrix africana* G.J. Marais & M.J. Wingf., *S. gemella* (Roets, Z.W. de Beer & P.W. Crous.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. palmiculminata* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. phasma* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. protearum* Marais & M.J. Wingfield, *S. protea-sedis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. splendens* G.J. Marais & M.J. Wingf., *S. variecibatus* Roets, Z.W. de Beer & P.W. Crous and *S. zambiensis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf. (Marais & Wingfield 1994, 1997, 2001 Roets *et al*. 2006, 2008, 2010). At least two additional undescribed species are known from this niche (Ngubane *et al.* 2017 (Chapter 4)). *Sporothrix* from *Protea* are not monophyletic (Ngubane *et al.* 2017 (Chapter 4)), indicating that this niche has been colonised more than once, with subsequent diversification in some lineages such as the *S. splendens* and *S. pulmiculminata* clades (Roets *et al*. 2006a).

The biology and dispersal ecology of some *Protea*-associated ophiostomatoid fungi are fairly well-studied. Mites are known to serve as primary spore-vectors of species such as *Knoxdaviesia proteae* (M.J. Wingf., P.S. Van Wyk & Marasas) Marais & M.J. Wingf. and *S. splendens* (Roets *et al*. 2006a, 2007, 2009). These mites achieve long-range dispersal between *Protea* populations through phoresy on nectar-feeding beetles (*e.g*. *Genuchus hottentottus* Fabricius and *Trichostetha fascicularis* L.; Roets *et al*., 2007, 2009b) and, as recently postulated, likely also on nectar-feeding birds (Aylward *et al.* 2014b, 2015; N. Theron-de Bruin *pers. comm*.). The relationship between *Sporothrix* and some mites in this system is mutualistic (Roets *et al.* 2007). For example, *S. splendens* is commonly isolated from *Trichouropoda* Berlese sp. mites found in the infructescences of *Protea repens* L. These mites can feed and reproduce on a diet consisting solely of *S. splendens* (Roets *et al.* 2007). In turn, the mites have special structures on their integuments for the transport of fungal spores while phoretic on beetles (Roets *et al.* 2007). Mites therefore act as the primary spore dispersers for *S. splendens*, whilst the fungus provides nutrition to the mites.

Sporothrix splendens is predominantly found in association with *P. repens* (Roets *et al.* 2009b). This host is one of the most widely distributed *Protea* species in South Africa and is found from the Northern Cape Province around Nieuwoudtville, throughout the Core Cape Region of the Western Cape Province (Manning & Goldblatt 2012) and extends into the Eastern Cape Province around Grahamstown (Rebelo 2001). It often forms dense stands throughout this distribution (Rebelo 2001). It is primarily pollinated by birds and insects (Coetzee & Giliomee 1985, Rebelo 2001) that are attracted to its large cream or pink inflorescences that form tightly closed seed-bearing infructescences after pollination (Figure 2.1). These infructescences can remain on the plants for several years (Bond 1984) and open to release seeds after the water connection between the plant and the infructescence is severed (usually after fire). This strategy is referred to as serotiny (Midgley & Enright 2000) and is observed in numerous CCR Protea species (Rebelo 2001). Closed infructescences house multitudes of fungi (Lee *et al.* 2005, Roets *et al.* 2006b), with *S. splendens* being one of the most prolific (Roets *et al.* 2009b).

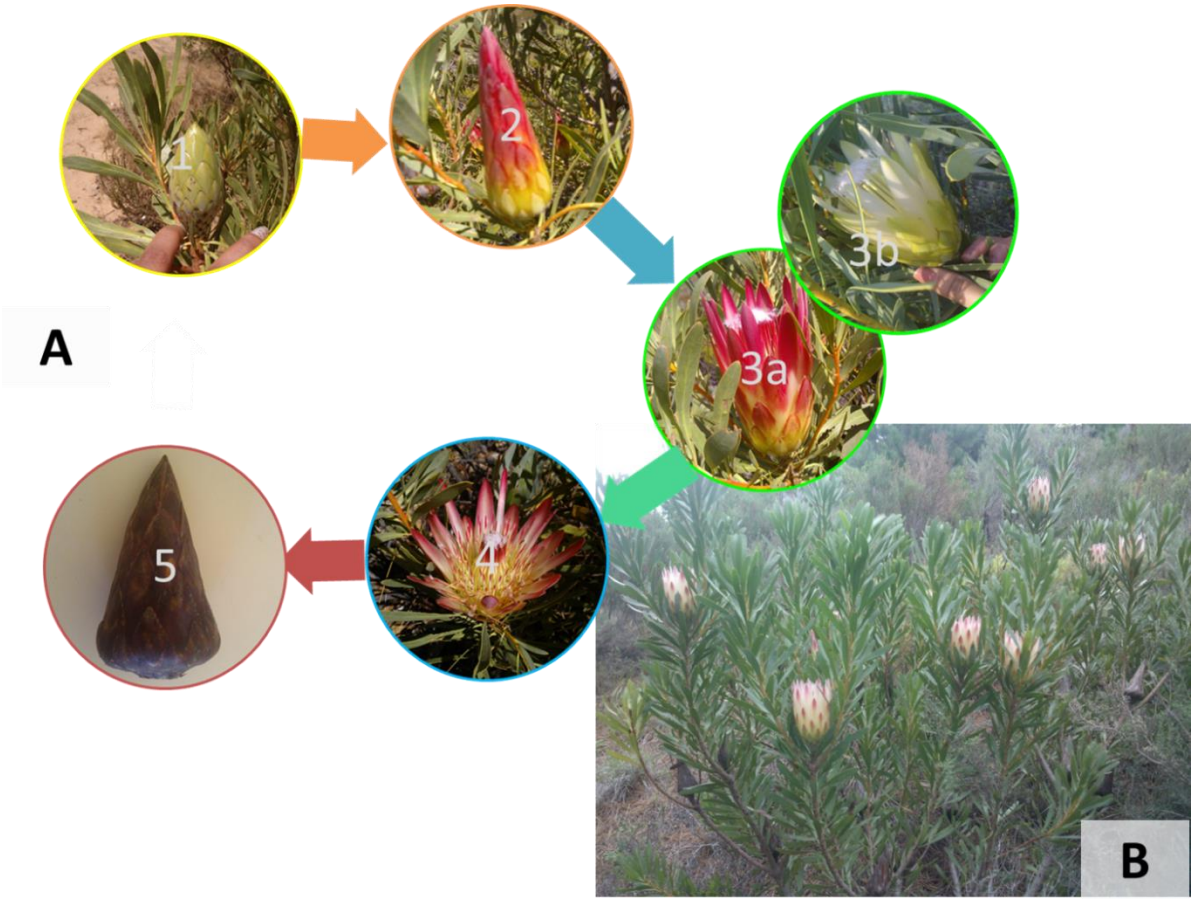

Figure 0.1:Developmental stages of Protea repens infructescences and inflorescences. (A) 1: young bud, 2: mature bud; 3: young inflorescence (3a: pink colour morph and 3b: cream colour morph); 4: fully opened inflorescence; 5: mature infructescence (B) Mature *Protea repens* plant. Photo credit: NP Ngubane

Although phylogenetically distant, *K. proteae,* exclusively associated with the infructescences of *P. repens* (Aylward *et al*. 2016, 2017), has a similar ecology and vectors to *S. splendens* (Roets *et al.*

2007, 2011). A recent population genetics study that used microsatellite markers to investigate the dispersal ecology of this fungus uncovered a near-panmictic population structure in two populations separated by approximately 240 km (Aylward *et al*. 2015). This led Aylward *et al*. (2015) to hypothesise that birds may act as additional vectors of these fungi, which raises interesting questions about the dispersal patterns and population structure of other ophiostomatoid fungi associated with *P. repens*, including *S. splendens*. Therefore, the aim of this current study was to investigate genetic diversity, gene flow, population structure and population differentiation of *S. splendens* across the entire geographical range of its *P. repens* host; and to compare this to what is known for *K. proteae*. It is expected that, due to the ecological similarities between these fungi, *S. splendens* will show near panmictic population structure over a very wide geographic range, and possibly throughout the extended range of the entire *P. repens* host.

1.6. Methods

1.6.1. Sampling design

Protea repens infructescences were collected from eight localities spanning its entire natural distribution range (from Nieuwoudtville in the Northern Cape Province to Grahamstown in the Eastern Cape Province; Figure 2.2). Between 40 and 60 one-to-two-year-old infructescences were collected per population from as many different *P. repens* trees as possible, with no more than three infructescences collected per individual tree. All infructescences were stored in paper bags at 10°C until fungal isolation.

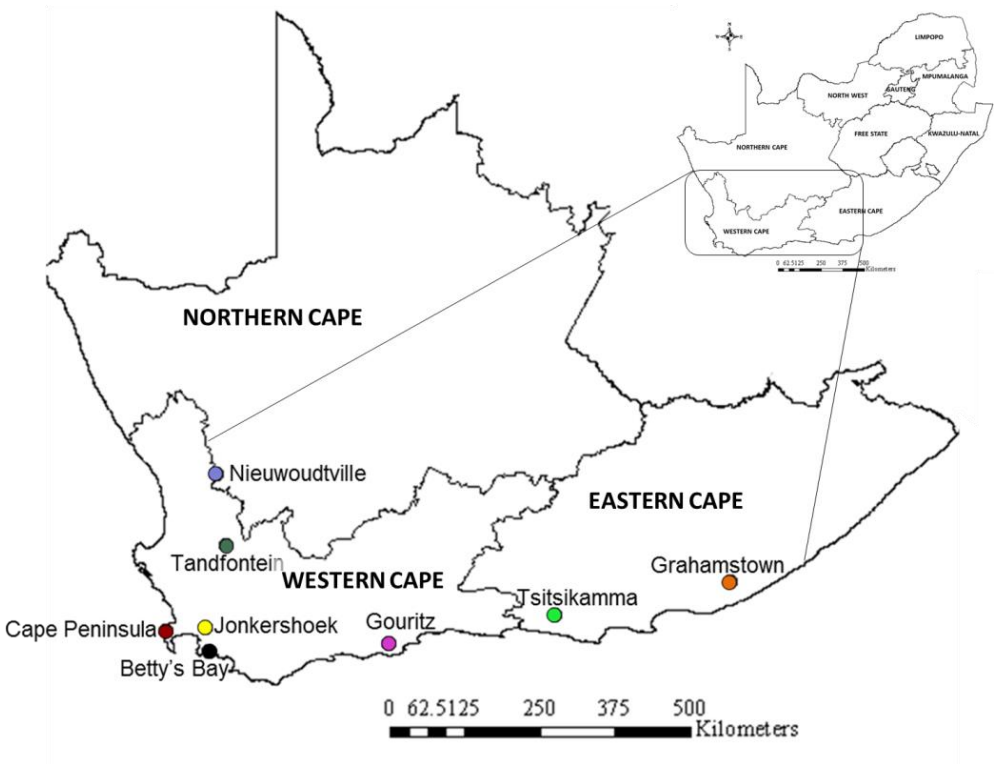

Figure 0.2: Distribution of the sampled populations of *P. repens* in South Africa. Colours reflect colours in the haplotype networks

1.6.2. Fungal isolation, culture and identification

Fungal isolation, DNA extraction and species confirmation followed methods outlined in Aylward *et al*. (2014a, b). Ascospores from single sporulating *Sporothrix splendens* ascomata (Figure 0.3) were collected using a sterile needle and transferred to Ophiostomatales-selective Malt Extract Agar plates (MEA; Merck, Wadeville, South Africa) containing streptomycin (0.04 g/L, Sigma-Aldrich, Steinham, Germany) and cycloheximide (2.5 g/L) (Roets *et al.* 2006a). The aim was to collect ten isolates per population for this study, with each isolate originating from a unique individual infructescence. Primary isolates were transferred to water agar (15 g agar/L; Merck, Wadeville, South Africa). Single pure cultures were obtained by transferring a single hyphal tip from the water agar to MEA plates and culturing these at 24°C in the dark until DNA extraction.

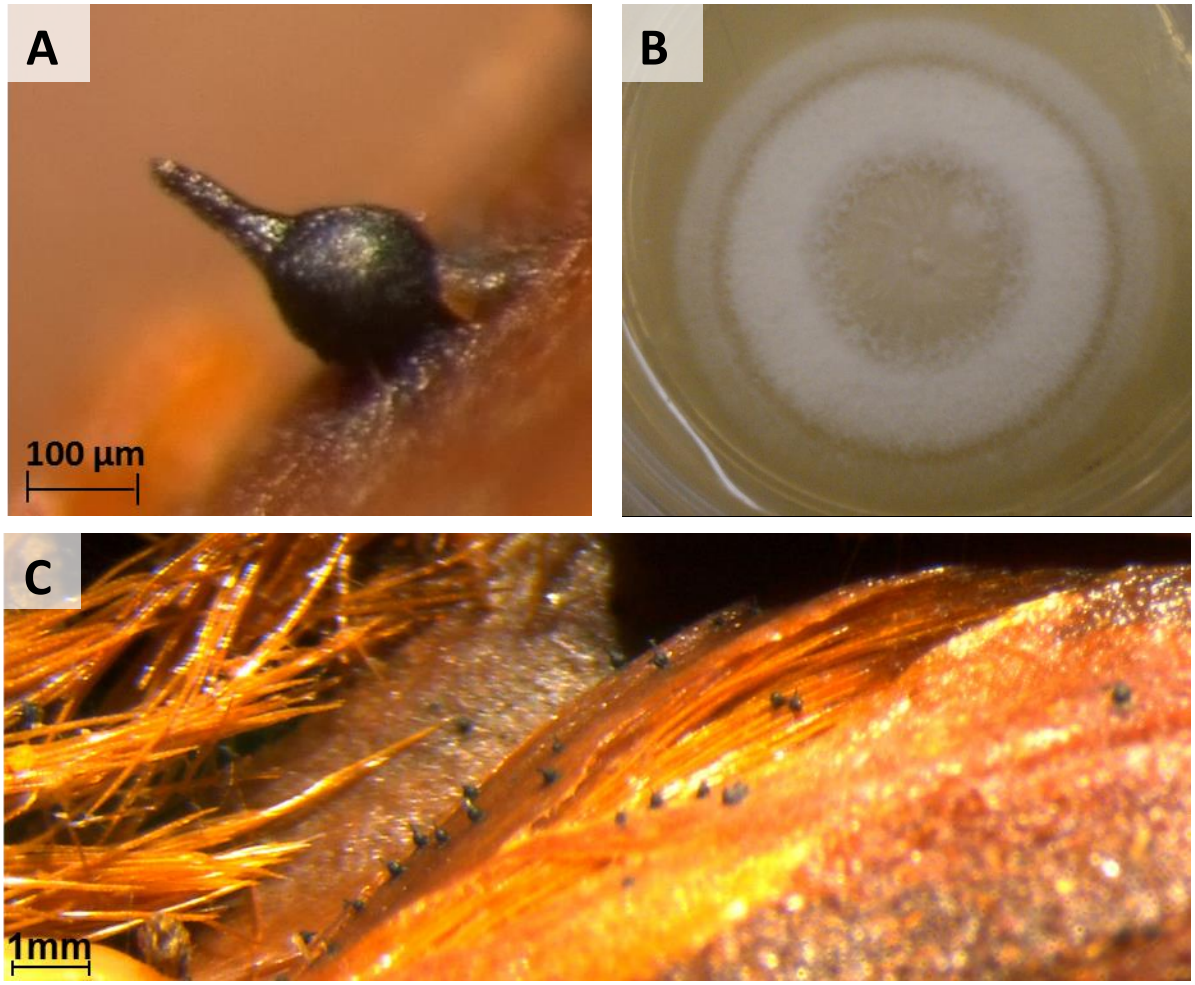

Figure 0.3: *Sporothrix splendens* morphology. (A) sexual morph, (B) asexual morph in culture (C) Multiple *S. splendens* sexual mophs on a single *P. repens* dead flower. Photo credit: NP Ngubane

For DNA extraction and purification, the CTAB protocol described by Möller *et al.* (1992) was followed with slight modification (Aylward *et al*. 2014a). Harvested fungal mycelia were placed in 2 ml eppendorf tubes containing 500 µl TES buffer (100mM Tris-HCl, pH 8.0; 10mM EDTA; 2% (w/v) SDS) (Bonello *et al*. 1998), 70 µg PCR grade Proteinase K (Roche Applied Science, Mannheim, Germany) and 4 glass beads (Möller *et al.* 1992). This solution was homogenised for 45 s at maximum RPM (30 cycles/s) using a tissue lyser (Qiagen Retsch, Walpole, MA, USA) and incubated at 55°C for 40 min.

For species identity confirmation, the Internal Transcribed Spacer (ITS) marker of a subset of individuals was amplified using the ITS-1F (5'-CTT GGT CATTT AGA GGA AGT AA-3') and ITS 4 (5'-TCC TCC GCT ATT GAT ATG C-3') primers (White *et al.* 1990, Gardes & Bruns 1993). Reaction mixes (25 µl per tube) contained 2.5 µl of 2.5 mM $MgCl_2$, 0.5 µl of a 10 µM stock of each primer, 12 µl 2X KAPA Taq ReadyMix PCR Kit (Kapa Biosystems, Inc., Boston, USA), 7.5 µl ddH_2O, and 2 µl of 100 ng/µl template DNA. PCR reaction protocols were: an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C

for 30 s, elongation at 72°C for 60 s, and termination with a final elongation step at 72°C for 8 min. PCR products were sequenced at the Central Analytical Facility (CAF), Stellenbosch University. The resulting sequences were compared to sequences of *S. splendens* strains from GenBank (http:// www.ncbi.nlm.nih.gov/blast/Blast.cgi) for species identity confirmation.

1.6.3. Sequencing of markers for population genetic analyses

For the purpose of this study two markers were chosen. A variable, but anonymous, marker located on contig 128 of the available genome sequence of a *S. splendens* isolate (CMW23050; *unpublished*) was amplified for population genetic analyses. This marker (m128) was initially targeted in a failed attempt to develop microsatellite markers for use in *S. splendens* population genetic studies (*unpublished data*), but showed enough variability between individuals of *S. splendens* to use in population genetic studies. The beta-tubulin marker (BT) was comparably less variable, but chosen as it contains numerous fast-evolving introns (4 exons and 3 introns (~230 bases) (Roets *et al.* 2009)) that may be informative at the population level (*e.g*. Gorton *et al.* 2004).

Amplification of the beta-tubulin marker was accomplished using the primers Bt2b (5'-ACC CTC AGT GTA GTG ACC CTT-3') (Glass & Donaldson 1995) and T1 (5'-AAC ATG CGT GAG ATT GTA AGT-3') (O' Donnell & Cigelnik 1997), and the newly developed primers m128F (AGG CGG CAT AGG TCT GTA ATA G) and m128R (TGG CAG TAT GAG CGA ATG AT) were used for the m128 marker. The beta-tubulin reaction mixtures were the same as those for the ITS marker. The reaction mixtures (20 µl) for the m128 marker consisted of 1 µl of 2.5 mM $MgCl_2$, 0.4 µl of each primer (10mM), 10 µl 2X KAPA Taq ReadyMix PCR Kit, 7.2 µl ddH_2O, and 1 µl of 100 ng/µl template DNA. PCR reaction conditions for beta-tubulin were: an initial denaturation step of 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 52.5°C for 90 s, and elongation at 72°C for 60 s, and a final elongation step at 72°C for 7 min. PCR reaction conditions for m128 were: an initial denaturation step of 3 min at 95°C, followed by 35 cycles denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 60 s, and a final elongation step at 72°C for 10 minutes. All PCR products were sequenced at CAF using the above specified PCR primers.

1.6.4. Data analyses

1.6.4.1. Genetic diversity

Base calling for sequences were verified using Chromas V.2.6 (Technelysium Pty Ltd, Tewantin, Australia) and the data sets were automatically aligned using Bioedit V.7.2.5 (Hall 1999), with subsequent manual adjustment. In order to determine the genetic diversity of *S. splendens* across all eight populations, various standard diversity indices were computed using DnaSP V5.10.01

(Librado & Rozas 2009) and Arlequin v3.5.2.2 (Excoffier *et al.* 2005). These included the number of haplotypes (n_h), haplotype diversity (h; Nei 1987; Excoffier & Lischer 2010), nucleotide diversity (π; Nei 1987) and various thetas (θ_H, θ_S, and θ_π). Haplotype diversity and nucleotide diversity measures are widely used to quantify the genetic diversity of many organisms including fungi (*e.g.* Abrinbana *et al.* 2010; de Jong *et al.* 2011; Tsui *et al.* 2012). Theta (θ) represents the ratio of the variance of allele frequencies among subpopulations to the overall variance in allele frequencies (Weir & Cockerham 1984). For sequence data, Tajima (1996) recommended using measures based on infinite-site models rather than infinite-allele models. We therefore calculated θ_S (based on infinite-site models) instead of θ_K (based on the infinite-allele models).

1.6.4.2. Population Structure

In order to visualise haplotype distribution between all *S. splendens* populations, haplotype networks were constructed for both markers using the Median-Joining algorithm in Network v.4. (Bandelt *et al.* 1999). We also included the haplotypes from the individual of *S. splendens* for which the genome was sequenced (CMW 23050). This isolate was collected from a *Trichouropoda* mite that was collected from Jan Marais Park, Stellenbosch (33° 55.976'S, 18° 52.603'E), a locality geographically close to the population that we sampled from Jonkershoek in this study (*ca*. 17 km).

1.6.4.3. Population Differentiation

Markers under positive selection are not well suited for studying population structure (Avise 2000). We therefore tested for neutrality in our data sets using Tajima's D (Tajima 1989), Fu's F_S (Fu 1997) and Ramos-Onsins & Rozas' R_2 (Ramos-Onsins & Rozas 2002) using Arlequin and DnaSP.

F_{ST} is the traditional descriptive statistic that measures genetic differentiation (Wright 1951). PhiST (Φ_{ST}; Excoffier *et al.* 1992) is an F_{ST}-related measure of genetic variation that incorporates a measure of genetic distance between alleles when measuring population differentiation. It does not assume that all mutation rates at different loci are equal and is best suited for sequence data (Excoffier *et al.* 1992, Holsinger & Weir 2009). This measure is also considered robust to high allelic diversity, unlike most fixation indices (Bird *et al.* 2011). Thus, Φ_{ST} values were calculated in this study using Arlequin based on models selected using jModelTest 0.1.1 (Posada 2008).

In order to evaluate variability among and within populations (or locations) Analysis of Molecular Variance (AMOVA) was conducted using Arlequin. A standard AMOVA was computed with a 10000 permutations and with a Kimura 2P model (significance level at 0.05) with a Gamma correction (m128: Γ= 0.260, beta-tubulin: Γ= 0.263) based on the model of evolution calculated using jModelTest. An exact test of population differentiation was conducted with the number of Markov chains set at 100 000 and the number of dememorization steps set at 10 000. The population from Nieuwoudtville was significantly different from the other seven populations with

Φ_{ST} values greater than 0.25 (classified as very great genetic differentiation by Hartl & Clark (1997)). Therefore, the AMOVA was repeated without the Nieuwoudtville population in order to determine to what degree this population was affecting overall population differentiation.

1.6.4.4. Gene flow between populations

To quantify the extent of gene flow between populations, the rate of gene flow (Nm) was calculated using DnaSP. Nm was calculated using Nei's G_{ST} (Nei 1973), Nei's Γ_{ST} (Nei 1982), and Hudson, Slatkin & Maddison's F_{ST} (Hudson *et al.* 1992). Nm calculated using Nei's G_{ST} (Nei 1973) was based on haplotype data, while the latter two were based on sequence data.

1.6.4.5. Isolation by distance

A Mantel test (Mantel 1967) was performed to determine whether there was any isolation by distance effect (IBD; Wright 1943) in *S. splendens* across the distribution of its *P. repens* host. Geographic distances between populations were calculated using a geographic distance matrix generator available online (http://www.geodatasource.com/distance-calculator) using GPS coordinates. Kimura 2P distance was calculated between all pairs of populations using Arlequin. The strength of correlation between genetic and geographical pair-wise distance matrices was tested using an Isolation by Distance Web Service (Jensen *et al.* 2005). A reduced major axis regression (RMA) line was used to calculate the intercept and slope of the genetic and geographic distance plot.

1.7. Results

1.7.1. Genetic diversity

Seventy-seven and 88 sequences were obtained from the beta-tubulin and m128 markers, respectively (with eight to 13 individuals per population). These sequences grouped into 51 and 38 haplotypes for the beta-tubulin and m128 markers, respectively. The beta-tubulin dataset included 870 characters of which 861 were usable (<5% missing data). There were 125 polymorphic sites, 43 sites with transitions, 26 sites with transversions and 67 sites with indels. The m128 dataset included 511 bases (all usable) and contained 80 polymorphic sites, 30 transitions, 16 transversions, 45 substitutions and 35 indels. Overall nucleotide diversity (beta-tubulin: 3.469% ($\pm$0.017) and m128: 2.044% ($\pm$0.010)) and haplotype diversity (beta-tubulin: 0.9839 ($\pm$0.0050) and m128: 0.966 ($\pm$0.0076)) was high for both datasets. High θ_H values for both markers indicated that the number of mutations per generation was high (Table 2.1). The average sequence divergence (θ_π) was much higher for the beta-tubulin marker than for the m128 marker (Table 2.1). Similarly, the expected number of segregating sites was higher for the beta-tubulin marker. Based on haplotype related measures and the θ measures, the beta-tubulin marker showed higher levels of genetic diversity than the m128 marker. Interestingly, beta-tubulin had a higher average number of

loci that were homozygous when compared in a pairwise manner (θ_H =59.084 (±19.020), Table 2.1).

Table 0.1: Different theta (θ) scores and their standard deviations for the beta-tubulin and m128 markers

	beta-tubulin	**m128**
Theta (H), θ_H	59.084(±19.020)	27.151(± 6.507)
Theta (S), θ_S	13.667(±3.807)	09.050(± 2.559)
Theta (pi), θ_π	29.864(±14.622)	10.520(± 5.360)

High haplotype diversity was evident across all sampled locations for both markers (Table 0.22.2), with the exception of Nieuwoudtville (m128: *h*=0.18182). Although haplotype diversity for the beta-tubulin marker in the Nieuwoudtville population was lower than the other populations, it was still fairly high (>0.5). The haplotype diversity using the beta-tubulin marker was highest in Betty's Bay (*h*=1.000) and for the m128 marker in Jonkershoek (*h*=0.964). The average number of differences between haplotypes (*k (h)*) was very high for all populations, except for Nieuwoudtville. Both markers showed high nucleotide diversity across all populations except for the Nieuwoudtville population (beta-tubulin: π= 0.200% (±0.001); m128: π= 0.590% (±0.004)). The highest nucleotide diversity was found in the Betty's Bay population for both markers (Table 2.2).

Table 0.2: Haplotype related measures for eight *S. splendens* populations based on the beta-tubulin and m128 markers

Marker	Location	*n*	n_h	*h*	π%	*k (π)*
Beta-tubulin	Nieuwoudtville	10	4	0.733(±0.101)	0.200(±0.001)	1.267(±0.868)
	Tandfontein	10	9	0.911(±0.077)	3.500(±0.019)	29.444(±14.089)
	Jonkershoek	10	8	0.956(±0.059)	2.146(±0.012)	18.111(±8.793)
	Cape Peninsula	10	7	0.911(±0.077)	3.540(±0.019)	32.667(±16.277)
	Betty's Bay	10	10	1.000(±0.045)	4.246(±0.023)	36.178(±17.235)
	Gouritz	8	7	0.964(±0.077)	3.080(±0.017)	25.750(±12.672)
	Tsitsikamma	8	6	0.893(±0.111)	3.503(±0.020)	29.393(±14.417)
	Grahamstown	10	7	0.867(±0.107)	2.813(±0.015)	23.267±11.203)
m128	Nieuwoudtville	11	2	0.182(±0.144)	0.590(±0.004)	2.909(±1.652)
	Tandfontein	13	8	0.923(±0.050)	1.141(±0.007)	5.577(±2.864)
	Jonkershoek	11	9	0.964(±0.051)	2.060(±0.012)	10.400(±5.143)
	Cape Peninsula	10	6	0.889(±0.071)	2.093(±0.012)	10.400(±5.143)
	Betty's Bay	12	6	0.848(±0.074)	3.164(±0.017)	15.788(±7.585)
	Gouritz	9	6	0.889(±0.091)	1.510(±0.009)	7.444(±3.843)
	Tsitsikamma	12	5	0.788(±0.090)	1.165(±0.007)	5.697(±2.937)
	Grahamstown	10	7	0.867(±0.107)	1.063(±0.006)	5.200(±2.749)

n = number of individuals; n_h = number of haplotypes; *h* = haplotype diversity; *π* = nucleotide diversity; *k(π)* = mean pairwise differences (calculated based on *π*)

1.7.2. Population structure

The m128 marker shared eleven haplotypes between populations and contained 27 private haplotypes (Figure 2.4A). The distribution of the haplotypes on the network did not reflect the geographic patterns of their source populations (Figure 2.4A). Six of the 11 shared haplotypes were shared between at least two populations from Jonkershoek, Tandfontein, Gouritz and Betty's Bay. Haplotypes congregated into two groups connected by at least two missing or ancestral haplotypes. The two haplotypes from Nieuwoudtville were unique to the sampling site, but they were distantly separated on the network. The beta-tubulin marker resolved to six shared haplotypes and 45 private haplotypes (Figure 2.4B). The network formed three branches (Figure 4B), but the distribution of haplotypes was also not reflective of the geographic origin of the haplotypes (Figure 2.2). In contrast to the m128 marker, the majority of haplotypes from the Nieuwoudtville population grouped close to each other. The m128 and beta-tubulin haplotypes of the isolate from the Jan Marais Park were the same as some of the haplotypes from Jonkershoek and Tsitsikamma, respectively (Figures 2.4A and 2.4B). The haplotypes from the sequenced *S. splendens* individual grouped with Jonkershoek (m128) and Tsitsikamma (beta-tubulin).

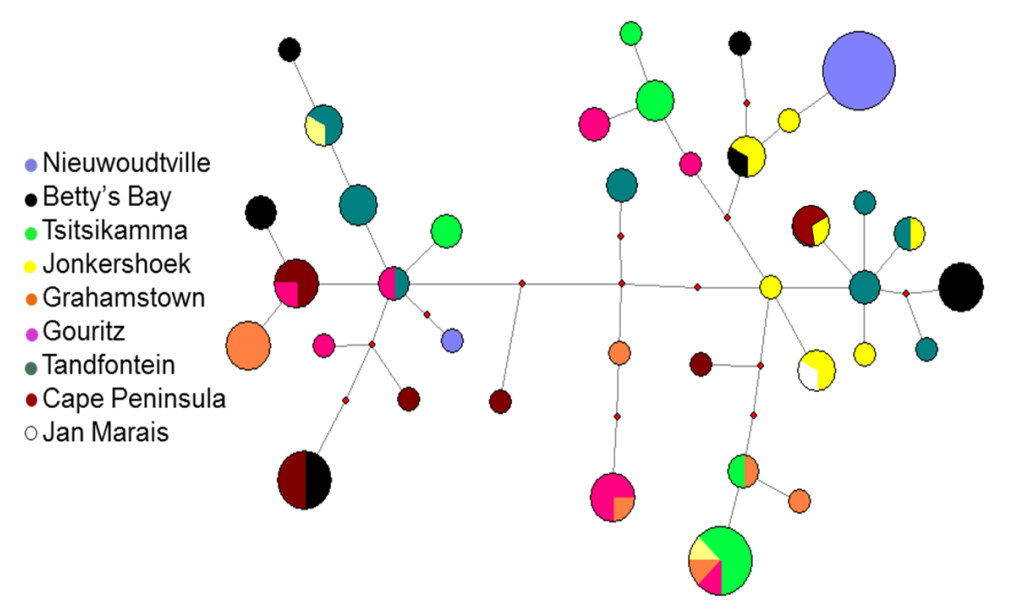

Figure 0.4A: Haplotype network of *Sporothrix splendens* using the m128 marker across eight sampled populations. Sizes of circles indicate the relative abundance of each haplotype (smallest = 1, largest = 10) and colours correspond to population of origin (Figure 2.2). The small red connections (median vectors) indicate hypothetical unsampled or ancestral haplotypes.

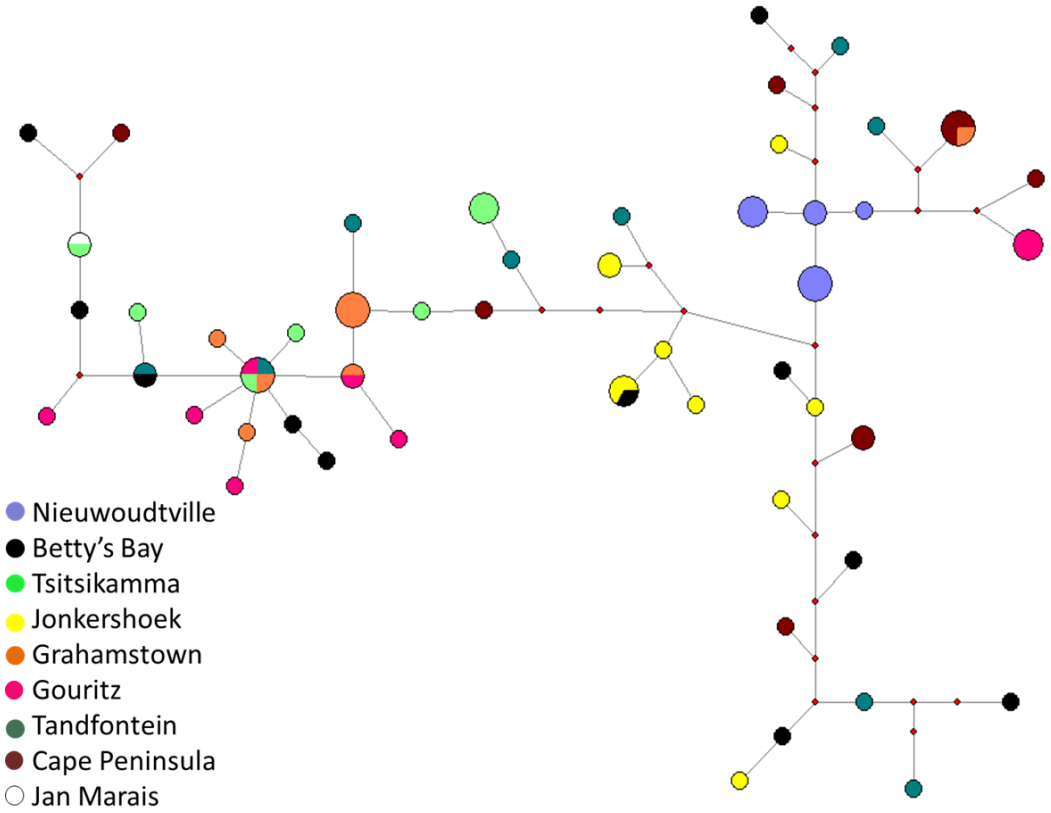

Figure 4B: Haplotype network of the beta-tubulin marker for *Sporothrix splendens* from the eight sampled populations. Sizes of circles indicate the relative abundance of each haplotype (smallest = 1, largest = 4) and the colours correspond to the population of origin (Figure 2.2) of the haplotypes. The red connections (median vectors) are hypothetical missing intermediates.

1.7.3. Population differentiation

The m128 marker was consistently found to be under no significant selection (Tajima's D= -0.857, p= 0.200; Fs= -7.115, p= 0.068; R_2 =009473, p=0.1550). Similarly, the beta-tubulin marker was not under significant selection (Tajima's D= 0.333, p= 0.688; Fs= -5.271, p= 0.122) with the exception of Roza's R_2 which suggested that it was under significant selection (R_2 =0.163, p=0.000).

All eight populations showed varying levels of differentiation when compared in a pairwise manner for both markers (Table 2.3). Interpretation of the Φ_{ST} values follow the guidelines provided by Hartl & Clark (1997) where values less than 0.05 indicate little genetic differentiation, between 0.05 and 0.15 indicate moderate genetic differentiation, between 0.15 and 0.25 indicates great genetic differentiation and values greater than 0.25 indicate very great genetic differentiation. The variation in the beta-tubulin marker across all eight populations revealed that Nieuwoudtville was significantly different from the rest of the populations (Φ_{ST}>0.25, p<0.05; Table 2.3). Nieuwoudtville was greatly differentiated from Betty's Bay (Φ_{ST} = 0.24389, p<0.05) and was very greatly differentiated from the other six populations (Φ_{ST}>0.25, p<0.05, Table 2.3), with greatest differentiation from the Grahamstown population (Φ_{ST} = 0.657, p<0.05). The Cape Peninsula population was very greatly differentiated from the rest of the populations, with the exception of Jonkershoek and Betty's Bay. Tandfontein ranged from very little to moderately differentiated from all populations with the exceptions of Nieuwoudtville and Cape Peninsula. Tandfontein and Tsitsikamma, Tandfontein and Grahamstown, Jonkershoek and Cape Peninsula, Jonkershoek and Tsitsikamma, Jonkershoek and Grahamstown, Cape Peninsula and Betty's Bay, Gouritz and Tsitsikamma, and Tsitsikamma and Grahamstown all had Φ_{ST} ranging between 0.05 to 0.15, indicative of moderate genetic differentiation between populations. Tandfontein and Jonkershoek, Tandfontein and Gouritz, Jonkershoek and Betty's Bay, Jonkershoek and Gouritz, and Gouritz and Grahamstown all had Φ_{ST} values less than 0.05, indicative of very little genetic differentiation (near panmixia).

Based on the m128 marker, genetic differentiation values ranged from moderate to very greatly differentiated between all groups sampled (Table 2.3). Much like the beta-tubulin marker, Nieuwoudtville was very greatly differentiated from all other populations (Φ_{ST}>0.25, p<0.05; Table 2.3). Tsitsikamma was also very greatly differentiated from Tandfontein, Jonkershoek and Betty's Bay. Tsitsikamma was most similar to Gouritz, much like for the beta-tubulin marker, although in this instance it was moderately differentiated (Φ_{ST} =0.070, p<0.05). The Cape Peninsula population was greatly differentiated from all other populations, with the exception of Tandfontein (Φ_{ST} =0.067, p<0.05) and Jonkershoek (Φ_{ST} =0.067, p<0.05). Grahamstown and Tsitsikamma populations displayed great levels of population differentiation, but were still more similar to each other than they were to other populations.

Table 0.3: Pairwise comparisons of the fixation indices, PhiST (Φ_{ST}), between all eight populations of *S. splendens* using the beta-tubulin (bottom of diagonal) and m128 (top of diagonal markers). All comparisons were significant (p<0.05).

	Nieuwoudtville	Tandfontein	Jonkershoek	Cape Peninsula	Betty's Bay	Gouritz	Tsitsikamma	Grahamstown
Nieuwoudtville		0.435	0.338	0.298	0.404	0.493	0.486	0.446
Tandfontein	0.518		0.067	0.104	0.108	0.300	0.381	0.258
Jonkershoek	0.462	0.001		0.121	0.053	0.243	0.297	0.178
Cape Peninsula	0.464	0.220	0.139		0.185	0.169	0.225	0.238
Betty's Bay	0.244	0.051	0.040	0.078		0.275	0.316	0.110
Gouritz	0.616	0.042	0.045	0.395	0.193		0.070	0.143
Tsitsikamma	0.636	0.119	0.102	0.414	0.228	0.071		0.206
Grahamstown	0.657	0.090	0.105	0.404	0.235	0.008	0.0524	

Based on the Analysis of Molecular Variance, 75.420% and 75.200% of the variation observed could be ascribed to variation within populations for the beta-tubulin and m128 markers, respectively (Table 2.4). Overall, there was great population genetic differentiation for both markers, (beta-tubulin: Φ_{ST}=0.246, p<0.05 and m128: Φ_{ST}=0.248, p<0.05). However, when Nieuwoudtville, the outlying population (Table 2.3), was removed from the analyses the variation ascribed to within populations increased considerably for both beta-tubulin and m128 to 85.7% and 82.66%, respectively (Φ_{ST}, decreased from 0.246 to 0.148 for the beta-tubulin marker and from 0.248 to 0.196 for the m128 marker).

Table 0.4: AMOVA results for population differentiation in *S. splendens* between eight populations based on the two markers (beta-tubulin and m128), with and without the Nieuwoudtville population (NWV) included.

Source of Variation	d.f.	S.S.	Variance	Percentage	Φ_{ST}	*p*-values
Beta-tubulin (inc. NWV)					**0.246**	**<0.005**
Among populations	7	187.572	2.134	24.580		
Within populations	68	445.160	6.546	75.420		
Beta-tubulin (excl. NWV)					**0.148**	**<0.005**
Among populations	6	118.155	1.301	14.880		
Within populations	59	439.177	7.444	85.120		
M128 (incl. NWV)					**0.248**	**<0.005**
Among populations	7	90.921	0.919	24.800		
Within populations	81	225.604	2.785	75.200		
M128 (excl. NWV)					**0.196**	**<0.005**
Among populations	6	71.966	0.745	19.610		
Within populations	71	216.960	4.0558	80.390		

Df: degrees of freedom; SS: sum of squares

1.7.4. Gene flow between populations

Nm values greater than one are indicative of gene flow between populations being high enough to negate the effect of genetic drift, whereas values greater than four indicate a panmictic population (Wright 1943). When the rate of gene flow for the beta-tubulin marker was measured using Nei's G_{ST}, Nei's Γ_{ST} and Hudson's F_{ST}, all indicated rates of migration significantly greater than one, which means that there was enough gene flow between populations to negate the effect of genetic drift (6.15 (G_{ST}= 0.068), 1.24 (Γ_{ST}= 0.136) and 1.64 (F_{ST}= 0.266), respectively). Similarly, under Nei's G_{ST}, Nei's Γ_{ST} and Hudson, Slatkin and Maddison 's F_{ST} rates of gene flow for the m128 marker were greater than one (2.38 (G_{ST}= 0.175), 1.66 (Γ_{ST}= 0.297), and 1.85 (F_{ST}=0.213), respectively). The high haplotypic diversity of beta-tubulin compared to that of m128 was reflected

in the haplotype data-based measure of Nm (Nei's Γ_{ST}) where the Nei's G_{ST}, based on Nm was much higher for beta-tubulin than for m128.

1.7.5. Isolation by distance

For the beta-tubulin marker, the Mantel test showed that there was no significant effect of distance between populations on genetic distance for the eight populations of *S. splendens* sampled here (r = -0.359, R_2 = 1.569e^{-04}, p = 0.5320; Figure 2.5A). There was a significant isolation by distance effect when using the m128 marker (r = -0.0125, R_2 = 0.176, p = 0.029; Figure 2.5B). However, these measures had very small r values, indicating that the observed patterns only accounted for approximately 1% of the given dataset. This shows a very weak correlation with a negative direction between these factors.

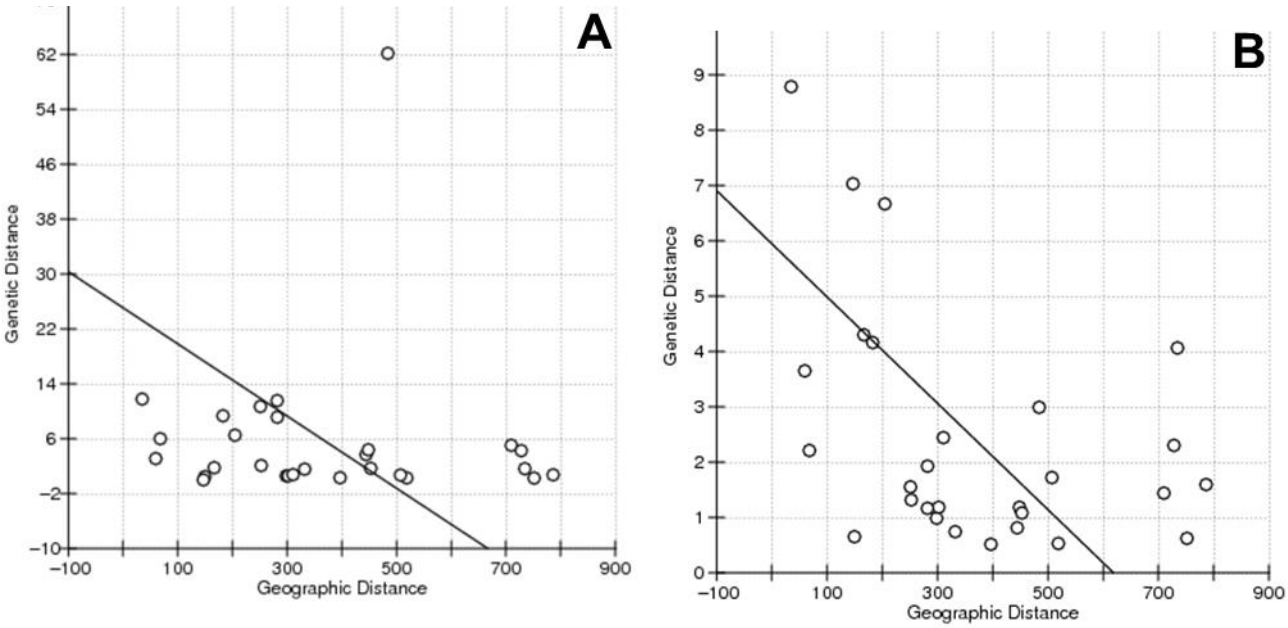

Figure 0.5: The isolation by distance plot of the genetic distance (Kimura 2P) *vs*. geographic distances (km) between populations using the beta-tubulin (A) and m128 (B) markers fitted to a reduced major axis (MRA) regression line.

1.8. Discussion

Results of this study show that genetically, individuals of *Sporothrix splendens* on *P. repens* do not group according to geography and that there is significant gene flow across all populations. Individuals from very distant populations also often share identical haplotypes. For example, populations from Grahamstown and Gouritz shared haplotypes (for both markers) and had very little to moderate genetic differentiation, even though these are separated by more than 450 km. This is indicative of frequent dispersal of fungal spores over very long distances. As the fungi grow and sporulate within the tightly closed infructescences and their spores are borne in sticky droplets attached to ascomata, wind and water dispersal of spores are unlikely in this system. Fungal

movement is therefore solely ascribed to the mites (primary vectors) via secondary vectors. Known secondary vectors include nectar-feeding beetles, but as pointed out by Aylward *et al*. (2015, 2016), movement of fungal spore-carrying mites on *P. repens* is likely enhanced by the involvement of birds as these beetles are infrequent in *Protea* inflorescences, they are not very active and their movement over vast distances would be impeded by geographical barriers.

Frequent outcrossing and high migration rates found in *S. splendens* would only be possible through effective spore dispersal (large numbers of propagules translocated to uncolonised niches). *Sporothrix splendens* is known to be phoretic on a *Trichouropoda* sp. (Roets *et. al.* 2007) and a *Tarsonemus* sp. (Roets *et al.* 2009a). Individual mites may carry numerous spores of *S. splendens* (Roets *et. al.* 2007). The number of mites phoretic on individual beetles can also be very high (averages of over 80 individuals per beetle have been recorded) (Roets *et al.* 20009a). Therefore, if ascospores are the main dispersal propagules as suggested by previous studies (Roets *et al.* 2007), and sexual reproduction stems from outcrossing as is the case for *K. proteae*, a visit by a single beetle to a uncolonised inflorescence should result in the transfer of numerous genetically distinct individuals that can provide genetically variable offspring. The involvement of birds on the long distance dispersal of these fungi (Aylward *et al.* 2015b), would explain the prevalence of haplotypes shared between distant populations of *S. splendens*. For example, populations from Grahamstown and Gouritz shared haplotypes (for both markers) and had very little (m128) to moderate genetic differentiation (beta-tubulin), despite the vast distance separating them.

Overall, the population structure of *S. splendens* did not match the population structure previously reported for other *Sporothrix* species which usually show grouping according to geography (Rodrigues *et al.* 2014a, Rangel-Gamboa *et al.* 2015). However, similarities between the population genetic structure of *S. splendens* presented here and that of the morphologically similar, but phylogenetically distantly related *K. proteae* are striking. For example, both fungal taxa have very high genetic diversity, show high rates of gene flow and, distant populations of both species collected in similar areas such as the Jonkershoek area (*K. proteae* was collected from Franschhoek in close proximity to Jonkershoek) and Gouritz, were nearly panmictic (Aylward *et al.* 2015b). *Knoxdaviesia proteae* shares the same *P. repens* host with *S. splendens* and at least one vector mite species (*Trichouropoda* sp.) that uses the same *G. hottentottus* beetle as a vector (Roets *et al.* 2007, 2009a, 2011). These two fungal species are also commonly found within the same infructescences, albeit growing in different areas. *Sporothrix splendens* is usually associated with the seeds close to the base inside infructescences, whereas *K. proteae* is found higher up on the decaying floral parts (Roets *et al.* 2006a). It is therefore unsurprising that, given their very closely matching ecologies, they show very similar population genetic structure. This suggests that the population structure of this species is not phylogenetically tethered, but rather driven by

convergent evolution. This does, however, raise interesting questions regarding the competitive abilities of these species. For example, what are the mechanisms that allow fungi with such closely matching morphologies and ecologies to co-exist in such a restrictive niche? It is possible that differential competitive abilities of the fungi based on differences in substrates and/or differences in interactions with possible mutualists (such as Actinomycetes (Human *et al.* 2016)) or other biota (such as fungal feeding mites) influence partitioning of this niche for the fungi. These aspects should be investigated in future studies.

Ascomata of *S. splendens* are very common within infructescences of *P. repens* indicating that sexual reproduction is a common phenomenon. The reason for this dominance in sexual reproduction may be ascribed to the need for production of ascospores over conidia in this ephemeral niche as ascospores are usually more resilient to desiccation and other strenuous conditions during dispersal (Aanen & Hoekstra 2007). Given the lack of recombination during the haploid phase (asexual phase) of *S. splendens*, such high genetic variability could be achieved through the addition of genetic variability that is only possible if the fungus cross-fertilises (Milgroom 1996, Moore & Novak Frazer 2002). The high genetic diversity of *S. splendens* can thus likely be ascribed to large population size and frequent outcrossing and recombination through sexual reproduction. It has been found that *K. proteae* is a heterothallic fungus and that outcrossing is therefore obligate (Aylward *et al.* 2016). Heterothallism is a common phenomenon in the Ascomycetes (Moser *et al.* 1995, Jacobs *et al.* 1998, Wilken *et al.* 2012, Duong *et al.* 2015) and may therefore also exist for *S. splendens*. In comparison to other *Sporothrix* species such as *S*. *schenckii*, the genetic diversity observed in *S. splendens* is much higher most likely due to the rarity of sexual reproduction in *S*. *schenckii* (Rangel-Gamboa *et al.* 2015, Teixeira *et al.* 2015).

Of all populations studied, the most isolated population found was that collected from Nieuwoudtville. This population was unique to our study in a number of ways; it was located close to the transition zone between the Core Cape Subregion and the Extra Cape region (Manning & Goldblatt 2012; Snijman 2013) and was collected from a small isolated population of fairly young *P. repens* plants. As recolonization after fire of *P. repens* by *K. proteae* was shown to largely depend on migration from neighbouring *P. repens* stands (Aylward *et al.* 2015b), a small, young and isolated host population such as the one sampled at Nieuwoudtville may be infrequently colonised by *S. splendens*. This would also explain the low number of haplotypes and low genetic diversity found in this population in our analyses.

In conclusion, *S. splendens* has high genetic diversity and levels of gene flow across the entire distribution range of its *P. repens* host. Population differentiation was generally moderate and population structure was low, supporting the findings of high levels of migration over vast distances shown by Aylward *et al.* (2014b, 2017). As *Protea*-pollinating birds are known to fly long distances

in search of flowering *Protea* stands (Fraser *et al.* 1989, Craig & Hulley 1994, Harrison *et al.* 1997), it is likely that they are involved with the dispersal of *S. splendens* as was hypothesised for *K. proteae* (Aylward *et al.* 2014b). Generally, patterns of population genetic structure and diversity of *S. splendens* closely matched that of the ecologically similar, but distantly related *K. proteae*. Future studies should determine whether *S. splendens* is a heterothallic species like its *K. proteae* counterpart and determine the mechanisms that allow these very similar taxa to co-exist in a very restricted niche.

1.9. References

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LARGE GEOGRAPHIC DISTANCE AND DIFFERENCES IN HOST IDENTITY DO NOT IMPEDE GENEFLOW BETWEEN *SPOROTHRIX* POPULATIONS ON *PROTEA* IN SOUTH AFRICAN GRASSLANDS AND SAVANNA

1.10. Abstract

The role of host selection and geographic locality are important factors that shape populations of species and may lead to speciation. In this study we investigated how these factors influence the population structuring of two very closely related species of ophiostomatoid fungi. *Sporothrix africana* and *S. protearum* are flower-associated fungi found on a variety of *Protea* spp. trees that occur throughout much of South Africa. These sister species share host plants and occur consistently throughout this distribution range which raise questions regarding reasons for their speciation. We calculated genetic diversity, population structure, population differentiation, migration and isolation-by distance between populations of these fungi on different hosts and over a large geographic range in order to clarify their dispersal ecology and evolution. Results revealed that these taxa can disperse over very long distances using insects and possibly birds as vectors. Populations did not segregate according to geography or host identity. We therefore found no evidence that these taxa represent two distinct species, but rather, that they likely represent morphological and genetic variants of a single, widely distributed, species.

Key words: convergent evolution, *Ophiostoma*, ophiostomatoid fungi, *Protea caffra*, *Protea dracomontana*, *Protea gaguedi*

1.11. Introduction

The genus *Sporothrix* (Z.W. de Beer, T.A. Duong & M.J. Wingf) Hektoen & C.F. Perkins contains more than 50 species and has a global distribution (de Beer *et al*. 2016). Members have varied ecologies that include saprobic soil-associates (Romeo *et al.* 2011), taxa associated with wounds on trees (Musvuugwa *et al.* 2015), arthropod-associates on trees (Musvuugwa *et al.* 2016) and pathogens of animals, including humans (*e.g.* Romeo *et al.* 2011, Kano *et al.* 2015; Rodrigues *et al.* 2015). In 1994, members of this genus were discovered in a very unusual habitat (Marais &

Wingfield 1994). They are exclusively associated with the infructescences of the African endemic plant genus *Protea* L. (Marais & Wingfield 1994). To date, eleven species have been described from this niche (Marais & Wingfield 1994, 1997, 2001, Roets *et al.* 2006a, 2008, 2010, Ngubane *et al.* 2017 (Chapter 4)). These species do not form a single monophyletic unit indicating multiple invasions of this niche (Roets *et al.* 2009b; Ngubane *et al.* 2017 (Chapter 4)). However, most *Protea*-associated species are confined to two clades, the *Sporothrix gemella* clade containing three species (*S. gemella* (Roets, Z.W. de Beer & P.W. Crous.) Z.W. de Beer, T.A. Duong & M.J. Wingfield, *S. palmiculminata* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingfield and *S. protea-sedis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer)) and the *Sporothrix splendens* clade that contains five species (*S. splendens* G.J. Marais & M.J. Wingfield, *S. protearum* Marais & M.J. Wingfield, *S. africana* G.J. Marais & M.J. Wingf., *S. zambiensis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *S. nsini sp. nov.*) (Roets *et al.* 2010; Ngubane *et al.* 2017 (Chapter 4)).

Protea-associated *Sporothrix* species rely on *Protea*-associated arthropods for dispersal (Roets et al. 2007, 2009a). As an example, *S. splendens* is an associate of *P. repens* L. in the Core Cape Subregion (CCR) of South Africa (Marais & Wingfield 1994). It is primarily dispersed by mites (Roets et al. 2007, 2009a) that live in a mutualistic association with this fungus (Roets *et al.* 2007). For long-distance dispersal the mites (and their associated fungi) rely on *Protea*-pollinating beetles such as *Trichostetha fascicularis* L. and *Genuchus hottentottus* Fabricius (Roets et al. 2006c, 2007) (Roets *et al.* 2006a, 2007, 2011). Recent evidence also suggests that *Protea*-pollinating birds are involved in dispersal of mites that carry *S. splendens* (Theron-de Bruin *et al. pers. comm*).

Most information about the ecology and dispersal of *Protea*-associated *Sporothrix* is based species associated with *Protea* hosts in the CCR. However, the distribution of the host genus extends from the CCR, through the northern parts of South Africa and into tropical Africa (Rebelo 2001). In South Africa, these northern *Protea* species host five *Sporothrix* species (Marais & Wingfield 1997, 2001, Roets *et al.* 2008) including two recently described species (Ngubane *et al.* 2017 (Chapter 4)). *Sporothrix africana* and *S. protearum* are very closely related sister species in the *S. splendens* clade (Roets *et al.* 2006a, 2010, de Beer *et al.* 2013, Roets *et al*. 2013, de Beer *et al.* 2016). These species were described based on morphological data (Marais & Wingfield 1997, 2001). They share *P. caffra* Meisn. as a host (Marais & Wingfield 1997, Roets *et al.* 2006a), but *S. africana* has also been recorded from other hosts such as *P. gaguedi* J.F. Gmel and *P. dracomontana* Beard (Marais & Wingfield 2001, Roets *et al.* 2006a). *Sporothrix protearum* was first collected from *P. caffra* infructescences from Drakensberg (KwaZulu-Natal Province), Ugie (Eastern Cape Province) and Pretoria (Gauteng Province) (Marais & Wingfield 1997). Both species

have been collected from *P. caffra* from Irene (Gauteng Province). The hosts of *S. africana* and *S. protearum* are also phylogenetically closely related (Valente *et al.* 2010). Therefore, it is unclear what barriers (if any) maintain the distinction between these species.

Northern *Protea* species such as *P. caffra* and *P. dracomontana* are pollinated by beetles (Steenhuisen *et al.* 2010, 2012b, Steenhuisen & Johnson 2012a, 2012b, 2012c) but are also frequented by birds (Downs & Perrin 1996, Calf *et al.* 2003a, Steenhuisen *et al.* 2012a). Although the ecology of their associated *Sporothrix* spp. remain poorly understood, it is suspected that they share the mite-mediated dispersal with their Core Cape Subregion (CCR) counterparts (Roets *et al.* 2009a). Also, the same *Protea*-pollinating beetle, *Trichostetha fascicularis* L., that has been implicated in the dispersal of *Sporothrix splendens*, pollinates some northern *Protea* species (Steenhuisen & Johnson 2012b). However, this species has been further classified into four subspecies, namely, *Trichostetha fascicularis fascicularis* L. (in the CCR), *Trichostetha fascicularis natalis* Burmeister (in Eastern Cape Province and north-western Lesotho), *Trichostetha fascicularis maraisi* Stobbia (in the north-western Northern Cape Province) and *Trichostetha fascicularis prunipennis* Burmeister (found in the Easter Cape Province, KwaZulu-Natal Province, Free State Province, North West Province, Mpumalanga Province, Limpopo Province and southern Botswana) (Holm & Perissinotto 2011). The distribution of these beetles largely does not overlap (Holm & Perissinotto 2011). Thus, if these subspecies are the main vectors for the mites (and their associated fungi) as is known in the CCR, it is likely that the population structure of *S. africana* and/or *S. protearum* will show the same geographic boundaries as the beetles. Although beetles have been identified as the main pollinators of northern *Protea* species (Steenhuisen *et al.* 2010, 2012b, Steenhuisen & Johnson 2012a, 2012b, 2012c), these proteas are also frequented by birds such as the greater double-collared sunbird, malachite sunbird and Gurney's sugarbird (Rebelo 2001). If birds are the primary dispersers of the mites (and the fungi) we expect that the fungi will be dispersed over much longer distances and that the genetic structure of *S. africana* and *S. protearum* will not reflect geographic location.

Population genetic studies have been conducted to investigate the dispersal ecology of various *Protea*-associated ophiostomatoid fungi (Wingfield *et al*. 1993) found in the CCR (Aylward *et al.* 2014a, 2014b, 2015b, 2017, Ngubane *et al.* 2017 (Chapter 2)). The dispersal of *S. splendens* on its *P. repens* host has been shown to be quite extensive (Ngubane *et al.* 2017 (Chapter 2)). For example, populations over 200 km apart were nearly panmictic, with some haplotypes shared between populations as far as 704 km apart (Ngubane *et al.* 2017 (chapter 2)). This is similar to another mite-dispersed fungus in *P. repens* infuctescences with similar ecology to *Sporothrix* from *Protea, Knoxdavesia proteae* M.J. Wingf., P.S. van Wyk & Marasas, that shows near genetic panmixia between populations over 240 km apart (Aylward *et al.* 2015b). A recent study on the

CCR endemic *Knoxdaviesia capensis* (M.J. Wingf. & P.S. Van Wyk) Marais & M.J. Wingf (Van Wyk & Wingfield 1993) showed that this fungus moves freely between multiple closely-related *Protea* hosts in the CCR (Aylward *et al.* 2017). The distinction between the sister species *K. proteae* and *K. capensis* is likely maintained by their differences in host preference (Roets *et al.* 2012; Aylward 2017). However, host identity as a barrier to maintain *S. africana* and *S. protearum* as separate species does not seem likely as they commonly share *P. caffra* as host and the reason for speciation between *S. africana* and *S. protearum* is therefore unclear. In order to gain insight into their population dynamics, more individuals from more populations and multiple hosts are needed.

The main aim of this study was to build on the meagre ecological knowledge of *Sporothrix* species associated with *Protea* hosts from the northern parts of South Africa and Eastern Cape. As species boundaries between *S. protearum* and *S. africana* are unclear, we assessed their taxonomic validity and explored the role of host identity and geographic distribution in shaping the populations of these taxa. We do this by investigating the genetic structure, diversity and gene flow between different hosts and across different geographic locations.

1.12. Methods

1.12.1. Sampling

Protea caffra has a patchy distribution from Hogsback (Eastern Cape Province) to Zambia and is considered one of the most widespread *Protea* species outside the CCR (Rebelo 2001). *Protea dracomontana* has a comparably confined distribution (a near KwaZulu-Natal Province endemic), found in a few locations including Underberg, Royal Natal, occurring throughout the Drakensberg National Park and extending towards to the Eastern Cape Province and KwaZulu-Natal Province border (Rebelo 2001). *Protea gaguedi* has a distribution that extends from the northern parts of KwaZulu-Natal Province (KZN) to Ethiopia (Rebelo 2001). *Protea dracomontana* and *P. gaguedi* often grow in sympatry with *P. caffra*.

Between 40 and 60 one-to-two-year-old infructescences of *Protea caffra*, *P. dracomontana* and *P. gaguedi* were collected per population from nine locations (Figure 3.1). Eight *P. caffra* populations were collected from locations extending from the Eastern Cape Province (Hogsback) to Renostepoort (Gauteng Province), with two of these from areas where they grow in sympatry with *P. dracomontana* (Drakensberg and Weza, KZN) and with one where they grow in sympatry with *P. gaguedi* (Renostepoort). As one of the type localities of *S. africana* (from *P. gaguedi*) was in the Blyde River Canyon (Marais & Wingfield 2001), this location was also sampled. Two of the sampled localities were type localities of *S. protearum* (Drakensburg Mountains (KZN) and Pretoria (Gauteng Province)). The Weza population and the Hogsback population were more than 150 km

away from the third type locality of *S. protearum*, Ugie (Eastern Cape Province). Infructescences were collected from as many different *P. caffra*, *P. dracomontana* and *P. gaguedi* trees as possible, with no more than three infructescences collected per individual tree. All infructescences were stored in paper bags in a fridge (10°C) until fungal isolation.

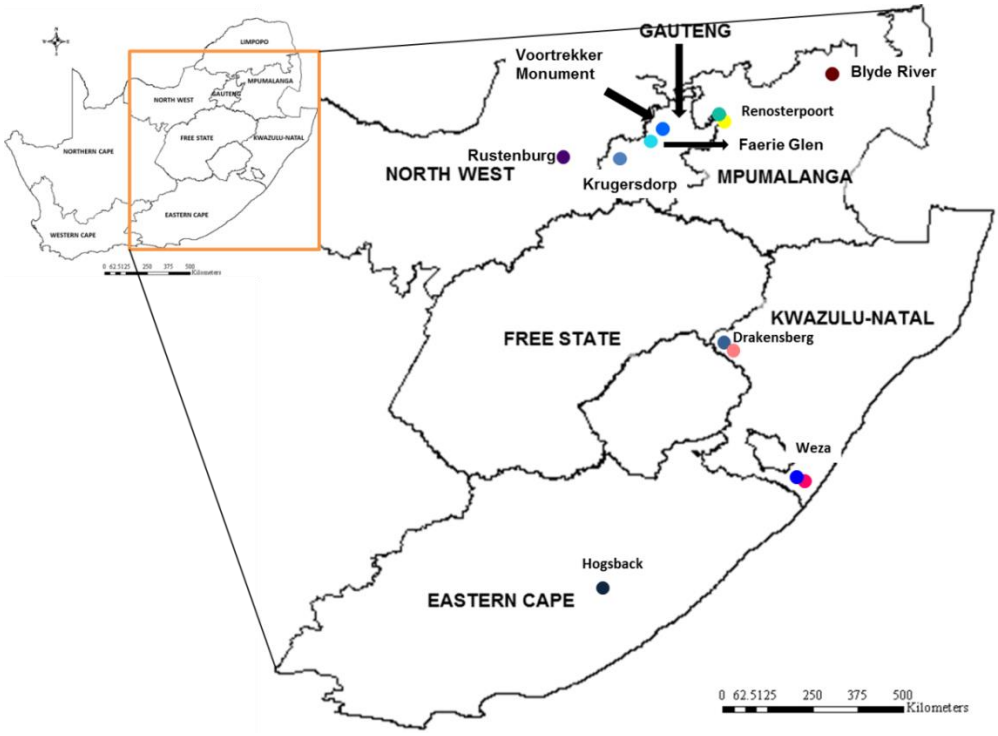

Figure 0.1: Distribution of the sampled populations in the Eastern Cape Province, KwaZulu-Natal Province, Gauteng Province, North West Province and Mpumalanga Province of South Africa. Blue, green and purple dots = *P. caffra*, pink and peach dots = *P. dracomontana* and yellow and brown dots = *P. gaguedi*.

1.12.2. Fungal isolation, culture and identification

Fungal isolation, DNA extraction and species identity confirmation followed methods outlined in Aylward *et al*. (2014a, b) and Ngubane *et al*. (2017, Chapter 2). Ascospores from single sporulating *S. africana* and/or *S. protearum* ascomata were collected using a sterile needle and transferred to selective media following the culture protocol used in Ngubane *et al.* (2017 (Chapter 2)). We did not try to distinguish between isolates of these two *Sporothrix* species as they are morphologically very similar, with most reported differences relating to very plastic characters such as hyphal ornamentation at the ascomatal base (Marais & Wingfield 2001). The aim was to collect ten *Sporothrix* isolates per host per population, with each isolate originating from a different individual infructescence. In order to confirm the identity of isolates collected and to see if there was any clustering according to species in the haplotype network, three confirmed isolates of *S. africana* and *S. protearum* were also included. These isolates were CMW1812 (*S. africana* from *P. dracomontana* from Drakensberg, KwaZulu-Natal Province), CMW 1107 (*S. protearum* from *P.*

caffra from Irene, Pretoria, Gauteng Province) and CMW 824 (*S. africana*, location and host identity unknown).

For DNA extraction, fungal mycelia were transferred into Eppendorf tubes and suspended in 500 µl TES buffer (100mM Tris-HCl, pH 8.0; 10mM EDTA; 2% (w/v) SDS), 70 µg PCR grade Proteinase K (Roche Applied Science, Mannheim, Germany) and 3-4 glass beads (Möller *et al.* 1992). This solution was mixed for 45 s at maximum RPM using a tissue lyzer (Qiagen Retsch, Walpole, MA, USA) and incubated for a 30 min to an hour at 55-60°C. The CTAB protocol described by Möller *et al.* (1992) was followed for DNA extraction and purification with slight modification (Aylward *et al*. 2014a).

Along with *S. africana*/ *S. protearum* other *Sporothrix* species such as *Sporothrix gemella* were also found within the sampled *Protea* species. Thus, to ensure that the cultured taxa belonged in the correct clade, the Internal Transcribed Spacer (ITS) marker of a subset of individuals were amplified using the ITS-1F (5'-CTT GGT CATTT AGA GGA AGT AA-3') and ITS 4 (5'-TCC TCC GCT ATT GAT ATG C-3') primers (White *et al*. 1990, Gardes & Bruns 1993). Reaction mixtures (25 µl per tube) contained 2.5 µl of 2.5 mM $MgCl_2$, 0.5 µl of each primer, 12 µl 2X KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA), 7.5 µl ddH2O and 2 µl of 100 ng/µl template DNA. PCR reaction conditions consisted of: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 60 s and termination with a final elongation step at 72°C for 8 min. Sequencing was done at the Central Analytical Facility (CAF), Stellenbosch University. The sequences were compared to *S. protearum*/*S. africana* sequences retrieved from GenBank (http:// www.ncbi.nlm.nih.gov/blast/Blast.cgi).

1.12.3. Sequencing of markers for population genetic analyses

To study the population structure of *S. protearum*/*S. africana* the extended beta-tubulin (~870 base pairs) and m128 markers were sequenced following the protocol in Ngubane *et al.* (2017 (Chapter 2)). Beta-tubulin reaction mixtures were the same as those used for the ITS marker. For the M128 marker, reaction mixtures (total volume of 20 µl) consisted of 1 µl of 2.5 mM $MgCl_2$, 0.4 µl of each primer (10mM stick), 10 µl 2X KAPA Taq ReadyMix, 6.8 µl ddH2O, and 1 µl of 100 ng/µl template DNA. PCR reaction conditions for beta-tubulin were: 94°C for 4 min (initial denaturation), followed by 35 cycles of 94°C for 60 s (denaturation), 52.5°C for 90 s (annealing), and 72°C for 60 s (elongation), and then a final elongation step at 72°C for 7 min. PCR reaction conditions for the M128 marker consisted of an initial denaturation step for 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 60 s, and a final elongation step at 72°C for 10 minutes. All PCR products were sent to CAF for sequencing.

Base calling of the resulting sequences was performed using Chromas V.2.6 (Technelysium Pty Ltd, Tewantin, Australia). These sequences were then aligned using Bioedit V.7.2.5 (Hall 1999).

1.12.4. Data analyses

Analyses were first conducted separating populations according to both host and geographic location such that, for example, fungi from Weza from *P. dracomontana* were considered one population and those from *P. caffra* from the same location were considered another separate population. Thereafter the analyses were repeated looking at populations defined by host such that all isolates collected from *P. caffra* were one population, those from *P. gaguedi* were a second population and those from *P. dracomontana* a third population. After this, analyses were also conducted comparing populations defined by geographic location where, for example, collections made from Drakensberg were considered one population regardless of whether they came from *P. caffra* or *P. dracomontana*.

1.12.4.1. Genetic diversity

In order to determine the genetic diversity (Moralez-Silva *et al.* 2014) of *S. protearum*/*S. africana* across all twelve populations for m128 and ten for beta-tubulin, various standard diversity indices were computed using DnaSP V5.10.01 (Librado & Rozas 2009) and Arlequin v3.5.2.2 (Excoffier *et al.* 2005). Haplotype diversity (also referred to as gene diversity; *h*; Nei 1987) provides information on the odds of alleles randomly picked in the sample being different (Excoffier & Lischer 2010), whereas nucleotide diversity (π) compares the average number of differences observed per site between sequences in a pairwise manner (Nei 1987). These measures are commonly used to assess diversity across a wide range of studies (*e.g.* Stumpf 2004, Tsui *et al.* 2012, Clarke *et al.* 2015) and were calculated here using Arlequin v3.5.2.2. All haplotype related measures we also calculated for location defined populations (regardless of host) and host defined populations (all fungi isolated grouped according to host).

The ratio of the variance of allele frequencies among subpopulations to the overall variance in allele frequencies is referred to as theta ((θ); Weir & Cockerham 1984). Different theta measures are implemented in population genetic studies of many fungal species as they are useful measures of genetic diversity of these organisms (Banke & McDonald 2005, Stukenbrock *et al.* 2006, Tsui *et al.* 2012). The overall mutation rate is measured using different molecular diversity indices such as theta (Hom), theta (S), theta (pi) and theta (K). Theta (Hom; θ_H) represents the number of mutations per generation computed using expected homozygosity (HomE). Theta (S; θ_S), estimated from the infinite-site equilibrium relationship, is a measure that estimates the rate of mutation per base between the number of segregating sites (Watterson 1975). Theta (θ_K) can be obtained from the observed number of alleles (K), and is based on the infinite-allele equilibrium relationship (Ewens 1972), that measures the relationship between the expected number of alleles

(k), the sample size (n) and θ. Theta (pi; θ_π), obtained from the mean number of pairwise differences (π), measures the average sequence divergence by calculating the number of nucleotide differences between two randomly chosen sequences from a population. Theta (*pi*) is the most useful when studying more historic expansion events whereas θ_S and θ_K are more informative and sensitive to recent events of expansion (Moralez-Silva *et al.* 2014). Tajima (1996) recommended measures based on infinite-site models rather than infinite-allele model for sequence data. Thus along with θ_S, θ_π was calculated using Arlequin.

1.12.4.2. Population Structure

To test the effect of geographic location populations were pooled according to the location from which they originate regardless of host and to test the effect of host association populations were pooled according to host disregarding the location from which they originate. Graphic representation of haplotype distribution of and the relationship between haplotypes across the landscape and on the different hosts was achieved by constructing median joining haplotype networks using Network v. 5.0.0.0 (Bandelt *et al.* 1999) for the two markers investigated.

1.12.4.3. Population differentiation

For studying population structure, using markers under positive selection is not recommended (Avise 2000). Thus, tests for neutrality were conducted by calculating Tajima's D, Fu's Fs and Ramos-Onsins & Rozas' R_2 (Ramos-Onsins & Rozas 2002), using Arlequin v3.5.2.2 (Excoffier *et al.* 2005) for the first two and DnaSP V5.10.01 (Librado & Rozas 2009) for the later. If populations are in violation of the mutation-drift equilibrium then Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) are expected to yield significant negative values with Fu's F_S yielding a larger negative FS values because it is very sensitive to population demographic expansion. Ramos-Onsins & Rozas' R_2 is better suited to use with small sample sizes (Ramos-Onsins & Rozas 2002). Neutrality tests were conducted first based on data separated according to host and geography, there after separated according to geography regardless of host and then according to host regardless of geography. However, the Ramos-Onsins & Rozas' R_2 could not be computed for large pairwise comparisons (grouped according to geography) due to restrictions posed by the programme (DnaSP).

F-statistics such as F_{ST} (Wright 1951) and F_{ST}-related measures (*e.g.* Φ_{ST} (Excoffier *et al.* 1992)) are used to measure population differentiation (Ceresini *et al.* 2007, Bird *et al.* 2011, Husseneder *et al.* 2013, Lee *et al.* 2016). F_{ST}, assumes that rates of evolution between alleles are equal (Balloux & Lugon-Moulin 2002), whereas, Φ_{ST} does not (Excoffier *et al.* 1992). Φ_{ST} also allows for an incorporation of a model that best fits the data at hand, and is therefore recommended for sequence data (Excoffier *et al.* 1992, Holsinger & Weir 2009). The best model describing the rate of nucleotide evolution for each data set was determined using jModelTest 0.1.1 (Posada 2008).

JModelTest yielded GTR models for both m128 and beta-tubulin (with Γ= 0.260 and Γ= 0.262, respectively) but as this model was not available in Arlequin v3.5.2.2, its closest relative, Kimura 2P (with gamma correction), was incorporated into Arlequin in order to compute Φ_{ST}.

Different approaches (for each marker) were employed to evaluate genetic variability within and among sample locations and hosts. First, pairwise comparisons between sampled locations and hosts were made using Arlequin. These comparisons were split three ways; 1: pairwise comparisons were done comparing populations separated according to host and geography (ten populations for beta-tubulin and 11 for m128), 2: pairwise comparisons were done comparing populations defined according to geographic location of origin (eight populations for beta-tubulin and nine for m128), and 3: pairwise comparisons were done comparing populations demarcated according to host (3 populations). Thereafter Arlequin was also used to conduct a global analysis of hierarchical molecular variance (AMOVA). This latter AMOVA partitions the total variance into covariance components by computing hierarchical distribution of variations among groups (F_{CT}), among populations within groups (F_{SC}) and within populations (F_{ST}). The hierarchical AMOVA was first set up such that the datasets was analysed grouped according to host (three groups) to test how population structure is influenced by host. In this instance, the different populations (split according to host and geography) were grouped on the basis of their hosts such that isolates from all isolates collected from *P. caffra* from different locations were considered one population, thus there were three populations with eight subpopulations (seven for beta-tubulin) in the case of *P. caffra*, two subpopulations with *P. dracomontana* and two subpopulations (one for beta-tubulin) in the case of *P. gaguedi*. Afterwards, the datasets were grouped according to locations (m128: nine and beta-tubulin: eight). Here, for beta-tubulin there were two geographic locations with two subpopulations each and for m128 there were three locations with two subpopulations each. These analyses were conducted using Arlequin using a Kimura 2P model with a gamma correction (as identified by jModelTest, following the Akaike Information Criterion) with significance level set at 0.05.

1.12.4.4. Gene flow

The extent of gene flow between populations was calculated for both markers by computing the rate of gene flow (Nm) using DnaSP V5.10.01. In this programme Nm was calculated using Nei's G_{ST}, Nei's Γ_{ST}, Hudson, Slatkin and Maddison's's F_{ST}. Nei's G_{ST} based Nm calculations only focus on the haplotype data whereas the latter two considers the complete dataset provided (including non-variable sites). The dataset was first analysed using data pooled according to host and geography and then using data separated according to host and geography.

1.12.4.5. Isolation by distance

We also determined if there was any significant isolation by distance effect between populations (IBD; Wright 1943). Geographic distances between populations were calculated using a geographic distance matrix generator available online (http://www.geodatasource.com/distance-calculator) using GPS coordinates of the sampled populations. PhiST-based genetic distances were calculated between all pairs of populations using Arlequin. The strength of correlation between the genetic and geographical pair-wise distance matrices using a Mantel test was tested using Isolation by Distance Web Service (Jensen *et al.* 2005).

1.13. Results

1.13.1. Genetic diversity

We obtained 69 beta-tubulin (49 from *P. caffra*, 14 from *P. dracomontana* and 5 from *P. gaguedi*) and 87 m128 sequences (63 from *P. caffra*, 17 from *P. dracomontana* and 7 from *P. gaguedi*). The limited sample size in some populations was due to the high prevalence of an undescribed species (Ngubane *et al.* 2017 (Chapter 4)) that morphologically closely resembled the target species. These sequences resolved into 40 and 67 haplotypes, respectively. The beta-tubulin data set included 843 bases (all usable, less than 5% missing data) of which 159 were polymorphic loci, 89 were transitions, 34 were transversions, 116 were substitutions, and 50 were indels. The m128 data set included 452 bases (all usable) and contained 194 polymorphic loci, 99 transitions, 69 transversions, 151 substitutions, and 49 indels.

Overall nucleotide diversity was 3.400 ($\pm$0.017) for the beta-tubulin data set and 3.700 ($\pm$0.018) for the m128 data set. Overall haplotype diversity was high for both data sets (beta-tubulin: h= 0.991 ($\pm$0.005); m128: h= 0.985 ($\pm$0.007)), with the exception of the subpopulation from Weza (*P. caffra*, h=0.417). Theta (H) was higher for the beta-tubulin marker (Table 3.1), indicating a higher level of homozygosity for the beta-tubulin marker than for the m128 marker. Haplotype diversity was consistently high across all sampled populations for both markers (h>0.5), and nucleotide diversity was consistently low (π <0.5) (Table 3.2). For the m128 marker, populations from Krugersdorp, Blyderiver, Drakensberg (*P. caffra*) and Rustenburg had maximum haplotype diversity (h = 1.000). The population from *P. dracomontana* at Weza had the lowest nucleotide diversity (π = 1.931), which was consistent with its low number of haplotypes (n_h=3) and lower haplotype diversity (h=0.417). For the beta-tubulin marker, populations from Krugersdorp, Blyde River Canyon, Drakensberg (*P. caffra* and *P. dracomontana*), Weza (*P. caffra*) and Rustenburg had maximal haplotype diversity (h=1). Of these populations, Drakensberg (*P. dracomontana*) had the highest nucleotide diversity and the highest average number of differences between haplotypes in the population (Table 3.2).

When grouped according to host and based on analyses of the beta-tubulin marker, *S. africana*/*S. protearum* populations from *P. gaguedi* had the highest haplotype diversity (*h*=1.000, Table 3.2). However, when using the m128 marker, populations from *P. dracomontana* had the highest haplotype diversity (*h*=1.000) but the lowest nucleotide diversity (π = 2.9) and the smallest average number of differences in the nucleotide diversity (k= 12.750). When grouped according to geographic localities the population from Drakensburg (both *P. caffra* and *P. dracomontana*) had had the second largest population size (*n*=12), highest number of haplotypes (n_h=12) and one of the highest haplotype diversity scores (*h*=1) when analysing data from the beta-tubulin marker (Table 3.2). When the m128 dataset was partitioned according to location, Krugersdorp, Blyde River Canyon and Rustenburg still had the highest haplotype diversity scores (*h*=1).

Table 0.1: Different theta (θ) scores for the beta-tubulin and m128 markers per host and location

		Theta (H), θ_H	Theta (S), θ_S	Theta (pi), θ_π
Beta-tubulin	Host			
	P. caffra	95.081	26.016	23.292
	P. dracomontana	12.660	18.867	18.981
	P. gaguedi	95.081	26.016	23.300
	Location			
	Hogsback	3.386	11.020	10.056
	Weza	10.887	4.822	3.959
	Drakensberg	—	21.193	21.359
	Krugersdorp	—	18.898	19.559
	Rustenburg	—	17.333	19.113
	Voortrekker Monument	—	19.501	21.162
	Faerie Glen	—	20.441	19.959
	Blyde River Canyon	—	18.720	19.732
M128	Host			
	P. caffra	99.867	29.709	16.568
	P. dracomontana	4.203	10.353	8.410
	P. gaguedi	—	12.245	12.750
	Location			
	Hogsback	3.666	26.746	31.451
	Weza	8.469	11.048	8.982
	Drakensberg	109.779	12.356	10.580
	Krugersdorp	—	13.432	12.819
	Rustenburg	—	10.000	10.574
	Voortrekker	20.730	11.267	11.144

Monument			
Faerie Glen	16.777	11.429	5.818
Renostepoort	1.414	7.333	8.226
Blyde River Canyon	—	10.949	12.304

—: scores could not be calculated because all the copies were different (heterozygosity high)

Table 0.2: Haplotype related measures for all *Sporothrix africana* and *S. protearum* populations based on the beta-tubulin and m128 markers for all populations, locations and hosts sampled

Marker	Location	*n*	n_h	*h* (±SD)	π% (±SD)	*k* (±SD)
Beta-tubulin	Hogsback C	7	4	0.810(+0.130)	1.600(±0.010)	13.524(±6.935)
	Krugersdorp C	8	8	1.000(+0.063)	2.545(+0.014)	21.250(±10.516)
	Blyde River Canyon G	5	5	1.000(±0.127)	3.002(+0.019)	25.100(±13.350)
	Weza C	8	8	1.000(±0.063)	0.930(±0.006)	7.750(±4.042)
	Weza D	8	4	0.786(±0.113)	0.763(±0.005)	6.321(±3.355)
	All Weza	16	11	0.925(±0.050)	0.500(±0.003)	3.959(±2.091)
	Drakensberg D	6	6	1.000(±0.10)	3.823(±0.023)	32.000(±16.346)
	Drakensberg C	6	6	1.000(±0.096)	3.377(±0.020)	28.133(±14.412)
	All Drakensburg	12	12	1.000(±0.034)	2.600(±0.014)	21.359(±10.147)
	Rustenburg C	3	3	1.000(±0.272)	2.700(±0.020)	22.000(±13.501)
	Voortrekker Monument C	9	7	0.917(±0.092)	2.900(±0.016)	24.167(±11.751)
	Faerie Glen C	8	6	0.893(±0.111)	2.885(±0.016)	24.143(±11.902)
	All *P. caffra*	49	42	0.990(±0.007)	2.800(±0.014)	23.292(±10.423)
	All *P. gaguedi*	5	5	1.000(±0.127)	2.400(±0.015)	19.715(±10.559)
	All *P. dracomontana*	14	10	0.934(±0.051)	2.300(±0.012)	18.974(±8.950)
M128	Hogsback C	13	5	0.821(±0.082)	5.300(±0.028)	23.680(±11.146)
	Krugersdorp C	10	10	1.000(±0.045)	3.200(±0.017)	11.652(±6.933)
	Renosterpoort	3	2	0.667(±0.314)	2.116 (±0.017)	9.333(±5.925)
	Blyde River Canyon G	6	6	1.000(±0.096)	2.900(±0.018)	12.800(±6.740)
	Weza C	9	8	0.972(±0.064)	4.783 (±2.648)	21.333(±10.412)
	Weza D	9	3	0.417(±0.191)	1.931(±0.011)	8.556(±4.370)
	All Weza	18	11	0.765(±0.108)	2.000(±0.011)	8.982(±4.340)
	Drakensberg D	8	7	0.964(±0.077)	3.167(±0.018)	14.000(±7.041)
	Drakensberg C	8	8	1.000(±0.063)	2.673(±0.015)	11.893(±6.031)
	All Drakensberg	16	15	0.992(±0.025)	0.024(±0.013)	10.580(±5.089)
	Rustenburg C	3	3	1.000(±0.272)	3.078 (±2.393)	13.667(±8.518)
	Voortrekker Monument C	11	9	0.946(±0.066)	2.822(±0.016)	12.418(±6.079)
	Faerie Glen C	7	6	0.952(±0.096)	3.077 (+0.018)	13.571(±6.958)
	All *P. caffra*	63	51	0.990(±0.006)	3.700(±0.018)	16.568(±7.476)
	All *P. dracomontana*	17	10	1.000(±0.076)	2.900(±0.017)	12.750(±6.557)

All *P. gaguedi*	7	7	0.838(±0.087)	1.900(±0.010)	8.410(±4.096)

n= number of individuals; n_h= number of haplotypes; *h*= haplotype diversity, π= nucleotide diversity; k= average number of differences between haplotypes in the population. Locations with a C, D and G at the end are locations of *Protea caffra* (C), *P. gaguedi* (G) or *P. dracomontana* (D).

1.13.2. Population Structure

The 67 m128 haplotypes resolved into 66 private haplotypes and one shared haplotype, and the 40 beta-tubulin haplotypes consisted of 38 private haplotypes and two shared haplotypes. The shared haplotype from the m128 marker was between isolates obtained from *P. caffra* and *P. dracomontana* from Weza (Figure 3.2B) while the two beta-tubulin shared haplotypes were shared between *P. dracomontana* and *P. caffra* in Weza, and between *P. caffra* from Faerie Glen and *P. caffra* from Voortrekker Monument.

Haplotypes did not cluster according to the locations from which they originate using either of the two markers (Figure 3.2Figure 0.4). Based on the beta-tubulin marker, haplotypes from Weza did form a cluster but this cluster also consisted of haplotypes from Hogsback and Drakensberg (Figure 3.2A). Haplotypes from the beta-tubulin sequences also did not cluster according to host (Figure 3.2A). Five of the 10 haplotypes from *P. dracomontana* clustered together but were also interspersed with haplotypes from *P. caffra.* Haplotypes from *P. gaguedi* were distributed across the network. The haplotypes originating from the m128 marker were evenly distributed across the network with no evidence of structuring according to host (Figure 3.2B). These trends were also evident when tracing haplotypes from the type localities for the two species, Drakensberg (*S. protearum*) and Blyde River Canyon (*S. africana*).

On the m128-based network, the haplotypes from the strains independently (of this study) identified as *S. africana* and *S. protearum* were separated by six connections (a Voortrekker Monument haplotype and 5 ancestral/missing haplotypes, Figure 3.2). Whereas, on the beta-tubulin- based network two *S. africana* isolates housed in the CMW collections were separated by 3 missing/ancestral haplotypes. CMW1812 (*S. africana*) was separated by three connections from the CMW1107 (*S. africana*) and from *S. protearum* (CMW824). Their close proximity in relation to the rest of the sampled haplotypes points to these individuals likely representing the same species.

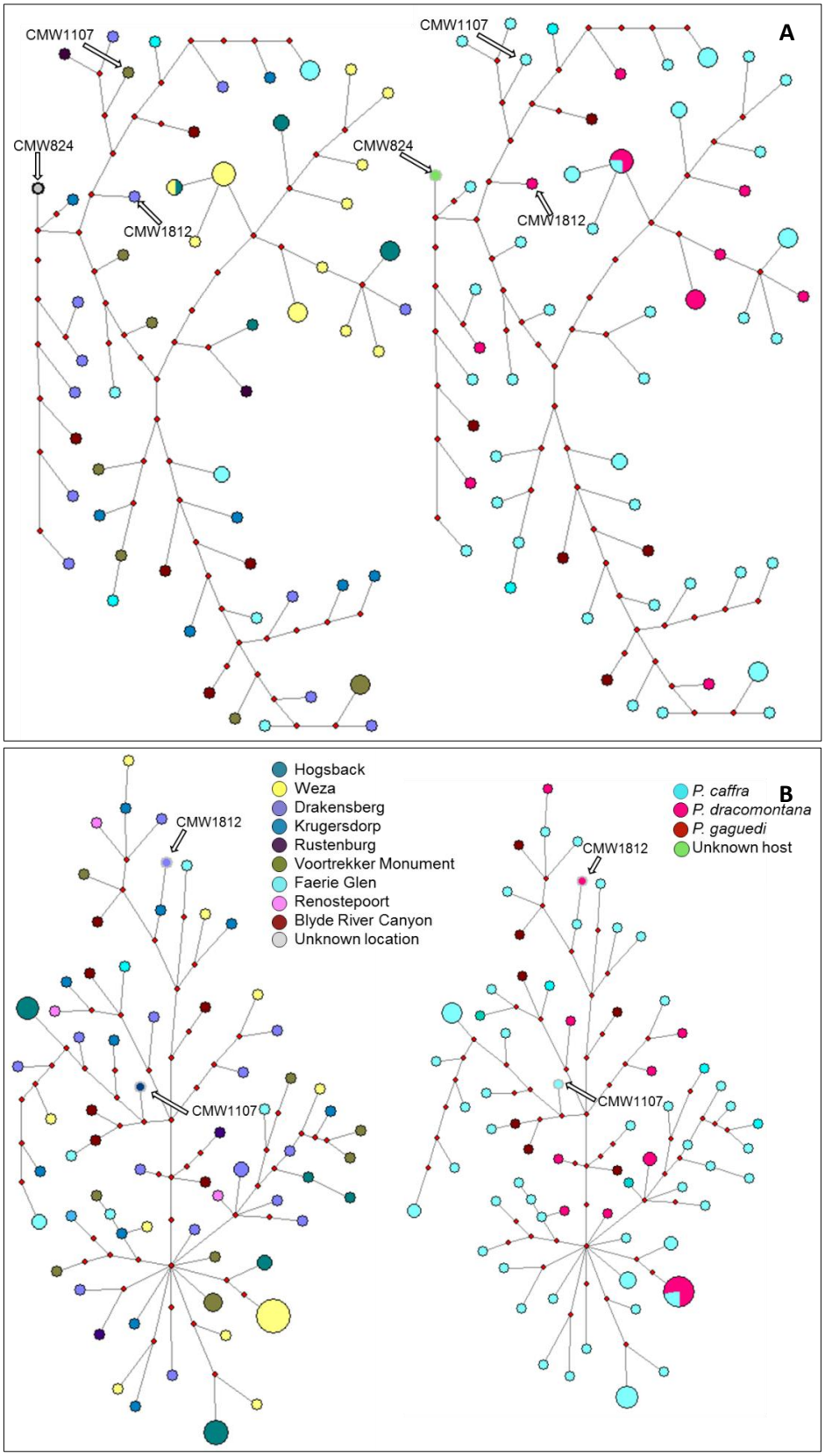

Figure 0.2: Haplotype network of *Sporothrix africana* and *S. protearum* based on the beta-tubulin marker (A) and the m128 marker (B) grouped according to geography (left) and host (right). Sizes of circles indicate the relative abundance of each haplotype (m128: smallest = 1, largest= 8; beta-tubulin: smallest=1, largest=4) and the colours correspond either the geographic origin of the population or the host identity from which the population originated. Haplotypes labelled with CMW numbers represent haplotypes from samples referred to as *S. protearum* (CMW1107,

(Roets et al. 2006c)) and *S. africana* (CMW824 (CMW Culture Collection) and CMW1812 (Roets *et al.* 2006a)) in the literature.

1.13.3. Population differentiation

When data were separated according to host and geography, Fu's F_S and Tajima's D consistently indicated that m128 was under significant negative selection (Tajima's D= -2.057, p= 0.002; F_S = -24.092, p=0) while Ramos-Onsins & Rozas's R_2 indicated that it was it under positive selection (R_2 =0.163, p=0). The Ramos-Onsins & Rozas's R_2 value was very small. In contrast, neutrality tests for the beta-tubulin marker indicated that there was no significant selection (Tajima's D= -0.827, p= 0.224; F_S= -14.643, p= 0.005), with the exception of the Ramos-Onsins & Roza's R_2 (R_2 =0.161, p=0.000). This test did however have a very small R_2 value. Similarly, for both m128 and beta-tubulin markers Fu's FS and Tajima's D were under no significant selections for different groups arranged according to geography (Appendix A). For neutrality tests (Fu's F_S and Tajima's D) performed on the dataset grouped according to geography, both markers were consistently under no significant selection across all nine groups (Appendix A). Similarly, the data grouped according to host identity indicated that both markers were under no selection with the exception of the Ramos-Onsins & Rozas R_2 that had small negative but significant values (Appendix A).

All 11 (for m128) and nine (for beta-tubulin) populations showed varying levels of differentiation when compared in a pairwise manner for both markers (Table 3.3). Interpretation of the Φ_{ST} values follow the guidelines provided by Hartl & Clark (1997) where values less than 0.05 means little genetic differentiation, between 0.05 and 0.15 means moderate genetic differentiation, between 0.15 and 0.25 means great genetic differentiation and values greater than 0.25 means very great genetic differentiation. Based on the m128 marker, when compared in a pairwise manner, populations ranged from near panmictic to greatly differentiated from each other (Table 3.3). Hogsback was near panmictic with all of the sampled populations, except with the Voortrekker Monument population from which it was moderately differentiated. Interestingly, isolates collected from distant populations of *P. caffra* from Krugersdorp and Drakensberg showed very little genetic differentiation and these were also not differentiated from Voortrekker Monument, Faerie Glen, Drakensberg (*P. dracomontana*) and Blyde River Canyon (*P. gaguedi*). Furthermore, they were both moderately differentiated from the populations collected from Weza (*P. dracomontana*) and Rustenburg (*P. caffra*). This suggests that the population collected from *P. caffra* from Krugersdorp was nearly identical to the one collected from Drakensberg based on this fixation index. The populations collected from Blyde River Canyon and Faerie Glen also showed very little genetic differentiation from each other. Both markers consistently showed that population from Voortrekker Monument and Faerie Glen were moderately differentiated (Table 3.3). Interestingly, the beta-tubulin marker showed that the population from Weza (*P. dracomontana*) was almost completely differentiated from most other populations (Φ_{ST}>0.05), while the m128 marker showed that it was almost panmictic with most other populations (Φ_{ST}<0.05). For the beta-tubulin marker, the Weza

(*P. dradomontana*) population was very greatly differentiated from all other sampled populations, with the exception of Hogsback and Weza (*P. caffra*) from which it was moderately differentiated.

Pairwise comparisons of populations based on the beta-tubulin marker from the eight locations were ranged from near panmictic to very greatly differentiated (Table 3.4). Blyde River Canyon, Krugersdorp and Rustenburg were near panmictic. *Sporothrix africana*/*S. protearum* from Hogsback and Weza were moderately differentiated from each other and very greatly differentiated from the other six populations. Interestingly, Voortrekker Monument, Faerie Glen and Krugersdorp were moderately differentiated from each other despite their close proximity. In addition, Drakensburg and Rustenburg were near panmictic (Φ_{ST}= 0.00, p<0.05). There was also a lack of geographic clustering when the m128 dataset of the populations were compared in a pairwise manor (Table 3.4). For example, distant populations such as Hogsback and Blyde River Canyon had very little differentiation from each other (Φ_{ST}= 0.024, p<0.05). Whereas, based on beta-tubulin, populations in close proximity such as Krugersdorp and Faerie Glen had very little differentiation (Φ_{ST}= 0.000, p<0.05). Rustenburg, on the other hand, had very little differentiation from Hogsback (Φ_{ST}= 0.013, p<0.05) while it was moderately differentiated from Voortrekker Monument (Φ_{ST}= 0.086, p<0.05) and Renostepoort (Φ_{ST}= 0.093, p<0.05).

Based on analyses of the m128 marker, pairwise Φ_{ST} comparisons showed that populations from *P. caffra* had very little genetic differentiation from *P. dracomontana* (Φ_{ST}=0.003, p<0.05) and *P. gaguedi* (Φ_{ST}=0.005, p<0.05). However, *P. dracomontana* was moderately differentiated from *P. gaguedi* (Φ_{ST}=0.056, p<0.05). In contrast, based on the beta-tubulin marker the three hosts were comparably more differentiated from each other than they were based on the m128 marker (Table 3.5).

Table 0.3: Pairwise comparisons of the fixation indices, Phi_{ST} (Φ_{ST}), between all populations (separated according to geographic location and host identity) using the beta-tubulin (above) and M128 (below) markers. All comparisons were significant (p<0.05)

	Hogsback C	Krugersdorp C	Weza C	Drakensberg C	Rustenburg C	Voortrekker Monument C	Faerie Glen C	Drakensberg D	Weza D	Blyde River G
Hogsback C		0.493	0.052	0.509	0.499	0.449	0.515	0.329	0.094	0.491
Krugersdorp C	0.030		0.605	0.000	0.000	0.078	0.055	0.061	0.612	0.000
Weza C	0.019	0.000		0.646	0.679	0.565	0.622	0.472	0.093	0.636
Drakensberg C	0.000	0.000	0.000		0.008	0.003	0.032	0.000	0.661	0.000
Rustenburg C	0.013	0.119	0.126	0.127		0.000	0.000	0.000	0.709	0.000
Voortrekker Monument C	0.058	0.017	0.035	0.000	0.086		0.091	0.026	0.571	0.074
Faerie Glen C	0.040	0.000	0.051	0.011	0.168	0.098		0.019	0.629	0.044
Drakensberg D	0.034	0.000	0.023	0.000	0.140	0.012	0.033		0.482	0.012
Weza D	0.045	0.078	0.064	0.088	0.236	0.097	0.170	0.113		0.652
Blyde River G	0.023	0.018	0.064	0.038	0.213	0.124	0.004	0.085	0.174	

Table 0.4: Pairwise comparisons of the fixation indices, Phi_{ST} (Φ_{ST}) grouped according to geographic location beta-tubulin (above) and m128 (below). All comparisons were significant (p<0.05).

				Voortrekker					
	Hogsback	**Krugersdorp**	**Rustenburg**	**Monument**	**Faerie Glen**	**Drakensberg**	**Weza**	**Blyde River**	**Renostepoort**
Hogsback		0.493	0.499	0.449	0.515	0.381	0.097	0.491	
Krugersdorp	0.030		0.000	0.078	0.055	0.029	0.675	0.000	
Rustenburg	0.013	0.119		0.000	0.000	0.000	0.742	0.000	
Voortrekker Monument	0.059	0.017	0.086		0.091	0.020	0.639	0.074	
Faerie Glen	0.040	0.000	0.168	0.098		0.027	0.691	0.044	
Drakensberg	0.051	0.000	0.136	0.006	0.030		0.561	0.001	
Weza	0.063	0.014	0.152	0.056	0.100	0.022		0.708	
Blyde River	0.024	0.018	0.213	0.124	0.004	0.071	0.108		
Renostepoort	0.000	0.017	0.093	0.011	0.096	0.029	0.085	0.150	

Table 0.5: Pairwise comparisons of the fixation indices, PhiST (Φ_{ST}) according to host for beta-tubulin (above) and m128 (below). All comparisons significant (p<0.05).

	P. caffra	*P. dracomontana*	*P. gaguedi*
P. caffra		0.070	0.036
P. dracomontana	0.003		0.256
P. gaguedi	0.005	0.056	

The global AMOVA indicated that 65.030% of the variation for the m128 marker was ascribed to variation within populations and 34.970% to among populations (Φ_{ST} = 0.34973, p<0.05). The results of the AMOVA ascribed 89.689% of the variation of the beta-tubulin marker to variation within populations and 10.311% to among populations (Φ_{ST} = 0.103, p<0.05).

The variation observed in *S. africana* and *S. protearum* could be explained by the variation within populations rather than based on host (group 1= *P. caffra* host, group 2= *P. dracomontana* host and group 3= *P. gaguedi* host; Table 3.6). For the beta-tubulin dataset, when the sampled populations were clustered according to the different hosts the AMOVA attributed 65.550% of the variation to variation within subpopulations regardless of the host from which they originated (Φ_{ST}=0.328, p<0.05; Table 3.6). The rest of the variation was attributed to the variation (34.450%) amongst hosts within subpopulations (Φ_{SC}=0.370, p<0.05). Based on the AMOVA for the m128 marker there was no structuring according to host and the majority of the variation (97%) was ascribed to variation within populations. However, this was not significant (Φ_{ST}=0.026, p=0.069, Table 3.6) and neither was the variation observed within subpopulations among hosts (Φ_{SC}=0.035, p=0.109, Table 3.6).

Hierarchical AMOVA analyses of the beta-tubulin dataset for the different geographic locations (Table 3.7) assigned 65.570% of the variations to variation within populations regardless of geographic location (Φ_{ST}=0.344, p<0.05), 34.25% to variation to among different locations (Φ_{CT}=0.343, p<0.05) and 0.18% to among populations within groups (Φ_{SC}=0.003, p=0.336). When hierarchical AMOVAs of the m128 marker were performed between locations (Table 3.7), the majority of the variation was attributed to within populations, 90.43% (Φ_{ST} = 0.096, p<0.05) and 5.090% was attributed to variation among populations within groups (Φ_{SC}= 0.053, p=0.05). Although 4.480% of the variation was attributed to among groups it was not significant (Φ_{CT}= 0.045, p= 0.392).

Table 0.6: Hierarchical AMOVA results for population differentiation between all populations for beta-tubulin (3) and M128 (3) grouped according to host.

	Source of variation	d.f.	sum of squares	Variance components	Percentage of variation	Φ-statistic (p-value)
M128	Among hosts	2	15.618	0.000	0.000	Φ_{CT}= 0.000 (p=0.695)
	Among hosts Within subpopulations	9	81.247	0.220	3.000	Φ_{SC}=0.035 (p=0.109)
	Within subpopulations	75	539.825	7.170	97.000	Φ_{ST}=0.026 (p=0.069)
	Total	**86**	**636.690**	**7.390**		
Beta-tubulin	Among hosts	2	49.271	0.000	0.000	Φ_{CT}= 0.000 (p=0.682)
	Among hosts Within subpopulations	7	274.301	4.000	34.450	Φ_{SC}=0.370 (p=0.000)
	Within subpopulations	58	450.136	7.000	65.550	Φ_{ST}=0.328 (p=0.000)
	Total	**67**	**773.708**	**11.000**		

Df= degrees of freedom

Table 0.7: Hierarchical AMOVA results for population differentiation between all populations for beta-tubulin (8) and M128 (9) grouped according to geographic location.

	Source of variation	d.f.	Sum of Squares	Variance components	Percentage of variation	Φ-statistic (p-value)
M128	Among locations	8	114.776	0.378	4.480	Φ_{CT}=0.045 (p=0.392)
	Among locations Within subpopulations	3	30.722	0.429	5.090	Φ_{SC}=0.053 (p=0.05)
	Within subpopulations	75	571.329	7.618	90.430	Φ_{ST}= 0.096 (p<0.05)
	Total	**86**	**716.828**	**8.42468**		
Beta-tubulin	Among locations	7	365.586	5.115	34.250	Φ_{CT}=0.343 (p=0.000)
	Among locations Within subpopulations	2	19.958	0.027	0.180	Φ_{SC}=0.003 (p=0.336)
	Within subpopulations	58	567.896	9.791	65.570	Φ_{ST}= 0.344 (p<0.05)
	Total	**67**	**953.441**	**14.93358**		

Df= degrees of freedom

When the rates of gene flow were measured using Nei's G_{ST}, Nei's Γ_{ST}, and Hudson, Slatkin & Maddison's F_{ST}, they all showed rates of migrations significantly greater than one indicative of enough gene flow to negate genetic drift, 4.610 (G_{ST} =0.098), 1.910 (Γ_{ST} =0.207) and 4.65 (F_{ST} =0.097), respectively, using the beta-tubulin marker. Likewise, using the m128 marker rates of gene flow were greater than one under Nei's G_{ST}, Nei's Γ_{ST}, and Hudson, Slatkin & Maddison's F_{ST}, 7.790 (G_{ST} = 0.060), 0.740 (Γ_{ST} =0.405), and 1.180 (F_{ST} = 0.298), respectively. The large difference between Nei's G_{ST} based Nm and the other two was because this measure was based only on haplotypic data while the other two measures considered entire sequences provided (Rozas *et al*. 2010). When compared in pair-wise manor, the rates of migration were high between all pairs of populations (defined based on geography) based on both markers (Appendix B). The population from Blyde River Canyon showed high rates of migration when compared to the other geographic locations with the lowest rates found when it was compared to the population from Weza, Nm=7.41. (Appendix B). Based on both markers the rates of gene flow (based on F_{ST} and Γ_{ST}) were lowest between populations sampled between *P. dracomontana* and *P. gaguedi* (Appendix B). Once again the G_{ST} based values were comparably higher than those obtained for F_{ST} and Γ_{ST}.

1.13.4. Isolation by distance

For the beta-tubulin marker, the Mantel test showed that there was no significant signal for isolation by distance between all the sampled populations of *S. africana* and *S. protearum* (R_2 =0.002, r= 0.047, p=0.560; Appendix C). Similarly, there was no significant isolation by distance effect when using the m128 marker (R_2= 8.266e^{-07}, r= 0.001, p= 0.485; Appendix C).

1.14. Discussion

The population structure of *S. africana* and *S. protearum* is not defined by either geography or host identity (supported by the lack of isolation by distance), and gene flow is characterised by high migration rates between subpopulations. Like other ophiostomatoid fungi from this unusual niche (*e.g. S. splendens*, *K. capensis* and *K. proteae*) the genetic diversity of *S. africana* and *S. protearum* is very high, indicative of high population size and frequent sexual recombination (Aylward *et al.* 2014b, 2015b, 2017, Ngubane *et al.* (Chapter 2)). There was no evidence that *S. africana* and *S. protearum* represent separate species based on population genetic measures as opposed to the limited molecular and morphological data used to separate these taxa in previous studies (Marais & Wingfield 1997, 2001). These two taxa therefore likely represent a single, freely interbreeding entity and should be considered a single species. Taxonomic nomenclature should therefore be updated to synonymise *S. africana* (described in 2001, Marais & Wingfield 2001) under *S.*

protearum (described in 1997, Marais & Wingfield 1997) after careful morphological and molecular consideration of all available isolates.

Sporothrix africana and *S. protearum* (hereafter referred to as *S. protearum*) can freely move and genetically recombine over extended distances (more than 900 km) as indicated by high genetic diversity and lack of population structure. The lack of geographic structuring was surprising as *Protea* spp. outside the CCR have a very patchy distribution (Rebelo 2001) and indicates that vectors of these entomochoric fungi must therefore also disperse frequently over vast distances. The vectors of *Sporothrix* that occur outside the CCR are unknown. However, as the dispersal ecology and population genetic structure of the closely related *S. splendens* is also characterised by frequent intermixing of populations over long distances (*c.a*. 700 km; Ngubane *et al*. 2017 (Chapter 2)), it can be assumed that the spore vectors of *S. protearum* are very similar to *S. splendens* from the CCR. *Sporothrix splendens* relies on various mites for primary dispersal, with *Protea*-pollinating beetles as secondary vectors (Roets *et al*. 2007, 2009). Pollinators of *P. caffra* and *P. dracomontana* (and likely also *P. gaguedi*) include beetles such as *Trichostetha fascicularis* (Steenhuisen & Johnson 2012b). This is the same beetle species, although a different subspecies, identified as one of the key vectors of *S. splendens* in the CCR (Roets *et al.* 2009a). However, given the large gap in the distribution between the delineated subspecies of *T. fascicularis* (Holm & Perissinotto 2011) the likelihood that this species is an important determinant of the population structure of *S. protearum* is unlikely. Similar to previous population studies on *Protea*-associated ophiostomatoid fungi (Aylward *et al.* 2014b, 2015b, 2017, Ngubane *et al.* (Chapter 2)), results of the present study therefore points towards the involvement of a dispersal agent that has much better dispersal capabilities than *Protea*-pollinating beetles. Most likely candidates in the CCR include orange breasted sunbirds and Cape sugarbirds (Aylward *et al.* 2015, Theron-de Bruin *pers. comm.*), species that are not found outside the CCR (Calf *et al.* 2003a, Chan *et al.* 2011).

Bird visitors of *Protea* spp. outside of the CCR include malachite sunbirds (Hargreaves *et al.* 2004) and Gurney's Sugarbirds (Calf *et al.* 2003a). Gurney's sugarbirds represent a different subspecies of the Cape Sugarbird and their distributions do not overlap (Calf *et al.* 2003a). However, their distribution ranges closely follow the distribution ranges of the *Protea* species that play host to two closely related *Sporothrix* species, one found in the CCR (*S. splendens* on *P. repens*, both confined to the CCR) and one outside it (*S. protearum* found on grassland and savanna *Protea* species in South Africa). This points either to a very close association between the birds and the fungi (possibly through the vectoring of *Protea*-associated mites), or similar ecological processes that shape the distribution of all taxa involved. It is known that the relationship between Gurney's sugarbirds and at least *P. caffra* is closely linked as the

timing of breeding in this nectar-feeding bird is aligned to the flowering period of *P. caffra* (de Swardt 1991, Calf *et al*. 2001). Thus, given the high frequency of their visitation on this *Protea* species (and likely the other two *S. protearum* hosts), it is expected that this bird plays a central role in *S. protearum* dispersal. The patchy distribution of *Protea* spp. outside the CCR and their occurrence in low densities (Acocks 1953) may encourage long distance travelling of the birds in search for nourishment.

Results of this study also indicated that *S. protearum* freely moves between different *Protea* host species as fungal populations from sympatric host populations were undifferentiated. This indicates that the spore-vectors (and pollinators) also move freely between these different *Protea* species that have the same phenology and pollinators (Downs 1997, Calf *et al.* 2003a, 2003b, Hargreaves *et al*. 2004, Steenhuisen *et al*. 2012b). Therefore, as was indicated in a study of *K. capensis* (Aylward *et al*. 2017), a *Protea*-associated fungus from the CCR with similar ecology to *S. africana*, pollination facilitation is likely to occur between sympatric *Protea* species (Rathcke & Lacey 1985, Rebelo 2001). This will also explain the high levels of hybridisation seen for these *Protea* species in the field (Rebelo 2001).

The high prevalence of missing/unsampled haplotypes in the haplotype networks suggests that there is a large number of unsampled variation in these populations, thus additional sampling would be needed to verify our findings with more robust data. Despite this, we still uncovered high haplotypic diversity for both markers (h>0.5), but low nucleotide diversity (h<0.5). This is comparable to that found in *S. splendens* populations across the CCR (Ngubane *et al.* 2017 (Chapter 2)). These patterns are indicative of a possible past population bottle- neck followed by rapid population expansion such as was recorded in other ophiostomatoid fungi such as *Ophiostoma montium* (Roe *et al.* 2011). These patters also suggest that *S. protearum* has a large population size with frequent outcrossing and recombination through sexual reproduction. In fact, ascomata of *S. protearum* are very common within infructescences of the *Protea* species investigated (nearly all infructescences contained numerous ascomata even though only a few contained fresh, sporulating structures), indicating that sexual reproduction is a common phenomenon. As with *S. splendens*, *K. capensis* and *K. proteae* from this ephemeral niche, this dominance in sexual reproduction may be ascribed to the need for production of more resilient ascospores over conidia to overcome strenuous conditions during long-distance dispersal events (Aanen & Hoekstra 2007).

In conclusion, *S. africana* and *S. protearum* likely represents a single species with remarkable genetic diversity. As was found for *K. capensis* in the CCR, a high level of gene flow and a high level of outcrossing likely prevent population differentiation, consequently

limiting the potential effects of genetic drift. This was irrespective of geographic location or differences in host taxa indicating a prevalence of long distance dispersal of fungal spores and a lack of discrimination between different *Protea* species by the spore vectors. Along with previous studies on these intriguing fungi (Aylward *et al.* 2014b, 2015b, 2017, Ngubane *et al.* (Chapter 2)), our study proposes a central role of birds in the dispersal of *Protea* flower-associated fungi, a process that has not yet been clarified.

1.15. References

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1.16. Supplementary Materials

Appendix A

Table 0.8: Fu's and Tajima's neutrality tests for each geographic location (beta-tubulin marker)

	Hogsback	Krugersdorp	Rustenburg	Voortrekker Monument	Faerie Glen	Drakensberg	Weza	Blyde River Canyon
Tajima's D	-0.919	-0.306	0.000	-0.160	-0.672	-0.462	-0.781	-0.329
p-value	0.213	0.395	1.000	0.456	0.268	0.350	0.233	0.473
Fu's FS	4.282	-0.820	1.963	2.157	2.825	-1.816	-1.513	0.783
p-value	0.963	0.200	0.529	0.814	0.888	0.115	0.224	0.408

Table 0.9: Neutrality tests (Fu's and Tajima's) for each geographic location for the m128 marker

	Hogsback	Krugersdorp	Blyde River Canyon	Weza	Drakensberg	Rustenburg	Voortrekker Monument	Faerie Glen	Renostepoort
Tajima's D	-1.186	-0.765	-0.047	-1.144	-1.002	0.000	0.000	-0.531	-0.308
p-value	0.107	0.234	0.503	0.122	0.158	1.000	1.000	0.321	0.402
Fu's FS	7.752	-2.569	-0.593	2.820	-4.567	1.469	4.053	-0.373	0.676
p-value	0.996	0.070	0.226	0.883	0.235	0.480	0.927	0.377	0.528

Table 0.10: Neutrality tests (Fu's F_S and Tajima's D) results for the *S. protearum* and *S. africana* populations from different *host species*

		P. caffra	*P. dracomontana*	*P. gaguedi*
M128	Tajima's D	**-1.992**	-1.120	-0.469
	Fu's F_S	**-24.081**	1.800	-1.097
	Ramos-Onsins and Roza's R_2	**0.160**	**0.162**	**0.161**
Beta-tubulin	Tajima's D	-0.751	-0.493	-0.329
	Fu's F_S	-8.154	2.134	0.783
	Ramos-Onsins and Roza's R_2	**0.161**	**0.163**	**0.163**

Appendix B

Table 0.11: Migration rate values (Nm) between pairwise populations (geographic) using the m128 marker based on different FST measures (G_{ST}, Γ_{ST} and F_{ST})

Location 1	Location 2	G_{ST}	Γ_{ST}	F_{ST}
Blyde River Canyon	Drakensberg	37.04	6.79	9.17
Blyde River Canyon	Faerie Glen	9.67	5.65	3.83
Blyde River Canyon	Rustenburg	36.0	1.74	2.06
Blyde River Canyon	Voortrekker Monument	27.05	4.03	4.58
Blyde River Canyon	Weza	7.41	4.34	2.52
Drakensberg	Faerie Glen	25.51	7.61	16.5
Drakensberg	Rustenburg	10.92	4.94	4.46
Drakensberg	Voortrekker Monument	29.36	11.56	5.8
Hogsback	Blyde River Canyon	7.86	3.63	2.9
Hogsback	Drakensberg	7.88	5.78	5.63
Hogsback	Faerie Glen	6.52	4.33	4.12
Hogsback	Krugersdorp	8.09	5.43	6.04
Hogsback	Mpumalanga	6.22	5.19	13.54
Hogsback	Rustenburg	6.22	3.58	2.43
Hogsback	Voortrekker Monument	6.22	3.58	2.43
Hogsback	Weza	3.48	4.08	2.89
Krugersdorp	Blyde River Canyon	12.00	5.69	14.43
Krugersdorp	Drakensberg	6.09	13.41	3.25
Krugersdorp	Faerie Glen	36.62	7.11	66.11
Krugersdorp	Mpumalanga	15.92	9.34	1.50
Krugersdorp	Rustenburg	15.92	3.60	4.17
Krugersdorp	Voortrekker Monument	35.43	7.93	27.38
Mpumalanga	Drakensberg	10.92	10.56	5.70
Mpumalanga	Faerie Glen	17.01	5.10	6.56
Mpumalanga	Rustenburg	2.01	1.62	6.41
Mpumalanga	Voortrekker Monument	11.31	6.39	8.74
Mpumalanga	Weza	5.83	6.52	6.35
Mpumalanga	Blyde River Canyon	36.0	4.47	2.18
Rustenburg	Faerie Glen	17.01	2.26	2.75
Rustenburg	Voortrekker Monument	11.31	4.1	6.04
Voortrekker Monument	Faerie Glen	17.55	4.52	5.79
Weza	Drakensberg	7.12	5.41	4.28
Weza	Faerie Glen	6.30	4.22	2.65
Weza	Rustenburg	5.83	4.68	2.04
Weza	Voortrekker Monument	5.95	4.44	3.11

Weza	Krugersdorp	7.48	4.68	3.43

Table 0.12: Migration rate values (Nm) between pairwise populations (geographic) using the beta-tubulin marker based on different FST measures (G_{ST}, Γ_{ST} and F_{ST})

LOCATION 1	LOCATION 2	G_{ST}	Γ_{ST}	F_{ST}
Blyde River Canyon	Drakensberg	37.04	6.79	9.17
Blyde River Canyon	Faerie Glen	39.67	5.65	2.83
Blyde River Canyon	Rustenburg	36	1.74	2.06
Blyde River Canyon	Voortrekker Monument	27.05	4.03	4.58
Blyde River Canyon	Weza	7.41	4.34	2.52
Drakensberg	Faerie Glen	17.27	7.41	18.27
Drakensberg	Rustenburg	13.33	7.53	65.29
Drakensberg	Voortrekker Monument	22.57	7.37	15.71
Hogsback	Blyde River Canyon	9.18	0.92	0.73
Hogsback	Drakensberg	9.69	1.42	0.76
Hogsback	Faerie Glen	5.74	0.78	0.49
Hogsback	Krugersdorp	9.55	0.94	0.61
Hogsback	Rustenburg	7.67	0.86	0.74
Hogsback	Voortrekker Monument	6.37	0.95	0.58
Hogsback	Weza	7.49	6.24	15.95
Krugersdorp	Blyde River Canyon	15.56	7.81	1.33
Krugersdorp	Drakensberg	240	6.99	13.03
Krugersdorp	Faerie Glen	17.67	5.03	10.2
Krugersdorp	Rustenburg	21.12	5.07	2.81
Krugersdorp	Voortrekker Monument	22.81	4.45	6.09
Rustenburg	Faerie Glen	10.7	5.05	2.6
Rustenburg	Voortrekker Monument	11.25	4.35	11.63
Voortrekker Monument	Faerie Glen	11.12	3.88	4.53
Weza	Drakensberg	25.08	0.79	0.5
Weza	Faerie Glen	10.11	0.72	0.5
Weza	Rustenburg	8.78	1.18	0.97
Weza	Voortrekker Monument	11.64	0.92	0.64
Weza	Krugersdorp	21.98	1.11	0.8

Table 0.13: Rates of migration (Nm) between host populations (populations grouped according to host species) based on three F-statistics for both markers

			G_{ST}	Γ_{ST}	F_{ST}
Beta-tubulin	*P. caffra*	*P. dracomontana*	23.31	10.21	4.89
	P. caffra	*P. gaguedi*	13.42	17.36	8.34
	P. dracomontana	*P. gaguedi*	16.66	2.06	1.31
m128	*P. caffra*	*P. dracomontana*	14.21	9.88	4.22
	P. caffra	*P. gaguedi*	18.92	18.36	6.49
	P. dracomontana	*P. gaguedi*	9.55	3.31	2.23

Appendix C

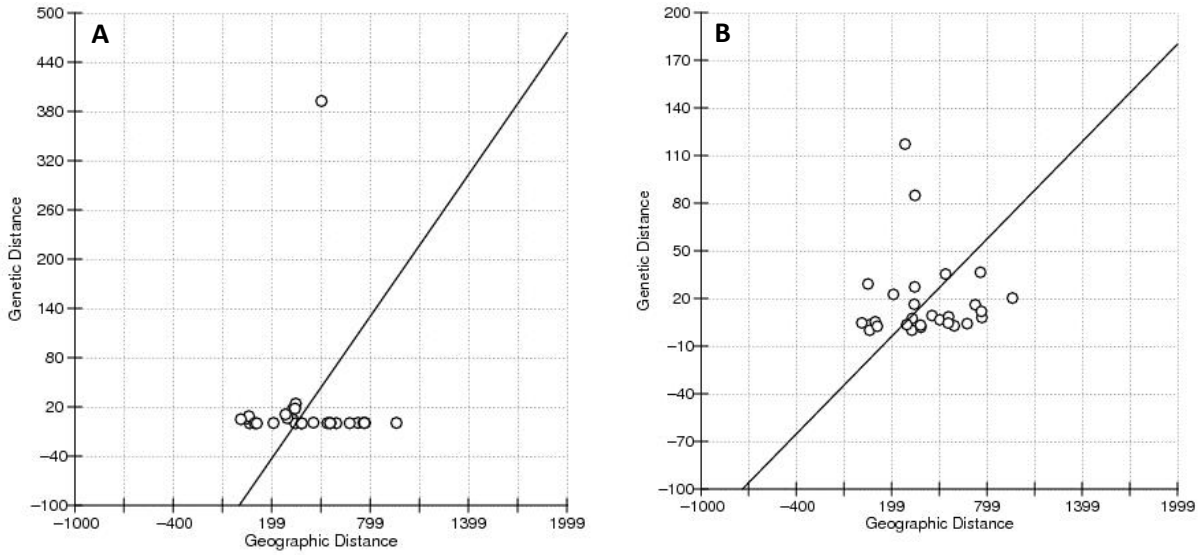

Figure 3.3: The isolation by distance plot of the genetic distance (Kimura 2P) against the geographic distances (km) between the populations (based on geographic location) using the beta-tubulin (A) and m128 (B) markers fitted to a reduced major axis (RMA) regression

TWO NEW *SPOROTHRIX* SPECIES FROM *PROTEA* FLOWER HEADS IN SOUTH AFRICAN GRASSLAND AND SAVANNA

1.17. Abstract

The inflorescences and infructescences of African *Protea* trees provide habitats for a large diversity of *Sporothrix* species. Here we describe two additional members associated with various *Protea* species from South African grasslands and savannas, raising the number of described species to eleven. *Sporothrix smangaliso sp. nov.* is distantly related to other *Protea*-associated species and, using multigene phylogenetic markers (ITS, beta-tubulin and calmodulin), groups with taxa such as *Sporothrix bragantina* from Brazil and *S. curviconia* from the Ivory Coast. *Sporothrix nsini sp. nov*. is sister to a clade containing four other *Protea*-associated species within the *Sporothrix stenoceras* complex. Both these newly described species share hosts with previously described species from this clade. This, and the collection of *Sporothrix nsini sp. nov.* from *P. caffra* infructescences where it was growing sympatrically with the closely related *S. africana* and *S. protearum*, highlights the need to study and understand the factors that influence host selection and speciation of *Sporothrix* in this atypical niche.

Key words: multigene phylogeny, new species, Ophiostomatales, ophiostomatoid fungi, *Sporothrix splendens*

1.18. Introduction

Accurately measuring fungal biodiversity is a daunting task, but is crucial to improve our understanding of global biodiversity (Zhou & Hyde 2001). Estimates of global fungal diversity ranges from 0.5 to 9.9 million species (Hawksworth 1991, Hammond 1995, Pascoe 1990, Rossman 1994, Cannon 1997, Arnold *et al.* 2000, O'Brien *et al.* 2005), but the most commonly used figure is around 1.5 million species (Hawksworth 1991). In South Africa, fungal diversity is conservatively estimated to include *ca*. 200 000 species, but only few native species have been described to date (Crous *et al.* 2006). A notable exception is the increasing rate of description of new southern African species in the Ophiostomatales (*e.g.* Kamgan *et al.* 2008, Roets *et al.* 2010, van der Linde *et al*. 2012, Musvuugwa *et al*. 2015, 2016, Osorio *et al*. 2016), a group that contains various plant pathogenic taxa (Tsui *et al.*

2012, Musvuugwa *et al.* 2015). The Ophiostomatales have infiltrated a range of niches, and have diverse ecologies ranging from human and plant pathogens (De Beer *et al.* 2003, Oliveira *et al.* 2014, Rodrigues *et al.* 2015) to saprobes (Lee *et al.* 2005, Roets *et al.* 2008). Their strong symbioses with other organisms further make them an appealing group to study (Six & Paine 1998, Zhou *et al.* 2001, Klepzig & Six 2004, Kolařík & Hulcr 2009, Six 2012, Yin *et al.* 2016).

Nine Ophiostomatales species in the genus *Sporothrix* (Z.W. de Beer, T.A. Duong & M.J. Wingf) Hektoen & C.F. Perkins (De Beer *et al*. 2016) have been described from an unusual niche, the flower heads of *Protea* L. (Proteaceae) shrubs and trees in South Africa (Marais & Wingfield 1994, 1997, 2001, Roets *et al.* 2006a, 2008, 2010). They are thought to be saprobic, and much research has accumulated on their general biology and ecology (Lee *et al*. 2005, Roets *et al.* 2005, 2006a, 2006c, 2009a, 2012, 2013). Much like their closely related relatives from conifers, *Protea*-associated *Sporothrix* rely on arthropods for dispersal (Klepzig *et al*. 2001, Roets *et al*. 2007, 2009a, 2011). They produce sticky spores that assemble at the tips of elongated perithecial necks from where they can easily attach to passing arthropods (Spatafora & Blackwell 1994). In the *Protea* niche, *Sporothrix* spores are primarily dispersed by mites belonging to genera such as *Thrichoropoda* Berlese and *Tarsonemus* Canestrini & Fonzago (Roets *et al*. 2007). These mites have specialised sporothecae, in which they store the fungal spores in transit (Roets *et al.* 2007). The relationship between at least some of the mites and the fungi is mutualistic, as the mites obtain nourishment from the *Sporothrix* species that they cultivate (Roets *et al*. 2007). These mites can traverse up and down the shrub, introducing spores to uncolonised flower heads from older fruiting structures (Roets *et al*. 2007). However, mites have limited dispersal abilities and thus require a vector for transport to more distant *Protea* plants and populations. This is achieved through phoresy on *Protea*-pollinating *Genuchus hottentottus* Fabricius, *Trichostetha fascicularis* L. and *T. capensis* L. beetles (Roets *et al.* 2009a). More recently, evidence has been mounting that birds are also involved in the dispersal of the mites that carry spores of *Protea*-associated fungi (Aylward *et al.* 2014a, 2015b) including *Sporothrix* (Theron-de Bruin *pers. com*.).

Phylogenetically, most *Protea*-associated *Sporothrix* species group in two clades (the *Sporothrix gemella* (Roets, Z.W. de Beer & P.W. Crous.) Z.W. de Beer, T.A. Duong & M.J. Wingf. clade and the *Sporothrix splendens* G.J. Marais & M.J. Wingfield clade) with two species (*Sporothrix variecibatus* Roets, Z.W. de Beer & P.W. Crous and *Sporothrix phasma* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf.) grouping outside of these two clades (Roets *et al*. 2013). The *Sporothrix gemella* clade contains *Sporothrix gemella*, *S. palmiculminata* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *S. protea-sedis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de

Beer, T.A. Duong & M.J. Wingf. (Roets *et al.* 2013). The *Sporothrix splendens* clade (the most speciose clade in this niche) consists of *S. africana* G.J. Marais & M.J. Wingf., *S. protearum* Marais & M.J. Wingf., *S. splendens* and *S. zambiensis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf. (Roets *et al.* 2013). Roets *et al*. (2013) also showed the existence of another, as yet undescribed species allied to *S. splendens* that in preliminary analyses resolved as sister to all other known species in this *Protea*-associated clade.

The concepts of host association, host specificity and exclusivity are important in understanding fungal diversity and biology (Zhou & Hyde 2001), and the *Protea*-associated *Sporothrix* species are no exception (Roets *et al.* 2012, 2013). Understanding how specific or exclusive fungi are to specific substrates or hosts can help better estimate the magnitude of fungal diversity (Zhou & Hyde 2001) and can be used to guide where research efforts should be directed in order to find the "missing" fungi (Hawksworth & Rossman 1997). For *Protea*-associated *Sporothrix*, host exclusivity (*i.e*. a saprobic fungus that exclusively occurs on a specific host or a on a restricted range of related host plants (Zhou & Hyde 2001)) or host-recurrence (the tendency of a fungus to be usually found on a specific host, but may also be encountered on other hosts in the habitat (Zhou & Hyde 2001)) varies between species. The reasons for this are unclear, but are thought to include vector relationships and host chemistry (Roets *et al.* 2012). *Sporothrix africana*, originally thought to only occur on *Protea gaguedi* J.F. Gmel. (Marais & Wingfield 2001), has since been isolated from *P. dracomontana* Beard and *P. caffra* Meisn. (Roets *et al.* 2006a). *Protea caffra* also hosts *S. protearum* (Marais & Wingfield 1997) and *S. gemella* (Roets *et al*. 2009b), both of which are thought to be specific to this host (Roets *et al*. 2009b). *Sporothrix protea-sedis* and *S. zambiensis*, described from Zambia, are also known from the single host, *P. caffra* (Roets *et al.* 2010). *Sporothrix palmiculminata* and *S. splendens* have been found on *Protea repens* (Roets *et al.* 2006a). Most other previous reports of *S. splendens* on other *Protea* hosts (*P. coronata* Lam, *P. lepidocarpodendron* L. and *P. neriifolia* Andrews) are thought to be of the more recently described *S. phasma* (Roets *et al.* 2009b).

Protea caffra is one of the most widespread *Protea* species in southern Africa, found from the Eastern Cape Province, through KwaZulu-Natal Province, Mpumalanga Province, Gauteng Province, Zimbabwe, all the way north into Zambia (Rebelo 2001). It often grows in sympatry with other *Protea* species such as *P. gaguedi* and *P. dracomontana* (Rebelo 2001). These closely related species (Valente *et al.* 2010) can easily hybridise (Rebelo 2001) in the field as they share pollinators (Steenhuisen & Johnson 2012) and therefore presumably also the vectors of associated *Sporothrix* species. In the course of a study that investigated the population dynamics of *S. africana* and *S. protearum* from various *Protea* species in South

Africa (Ngubane *et al.* 2017 (Chapter 3)), two apparently undescribed *Protea*-associated *Sporothrix* species were encountered. One of these had identical ITS sequences to the undescribed *Sporothrix* species first documented by Roets *et al.* (2009b) from *P. caffra* in the Walter Sisulu National Botanical Gardens, Gauteng Province. The additional material collected in the present study made it possible to evaluate the specific status of this taxon based on molecular and morphological data as well as additional distribution and host-association data. The second apparently undescribed species was sporadically isolated from various hosts from KwaZulu-Natal Province. Preliminary analyses based on internal transcribed spacer (ITS) data suggested that it is quite distinct from all other known *Protea*-associated species. In the present study we therefore also evaluate the specific status of this taxon in terms of morphology, DNA phylogenetic data and ecology.

1.19. Methods

1.19.1. Sampling

Infructescences of *Protea* species were collected from various sites across South Africa including: *P. caffra* from Faerie Glen Municipality Nature Reserve (Gauteng Province, 25° 46.360'S, 28° 17.878'E) and Voortrekker Monument (Gauteng Province, 25° 46.625'S, 28° 10.521'E); *P. dracomontana* from the Royal Natal Nature Reserve in Drakensberg (KwaZulu-Natal Province, 28° 50.185'S, 28° 56.902'E), and *P. gaguedi* from Bivane Dam in Vryheid (KwaZulu-Natal province, 27° 33.450'S, 31° 3.970'E) and Blyde River Canyon (Mpumalanga Province, 24° 37.300'S, 30° 48.860'E).

1.19.2. Morphological characterization

Morphological structures of both putative new taxa were studied using specimens mounted in clear lactophenol or lactophenol with cotton blue (Sigma-Aldich, Germany) on microscope slides and viewed on a Leica EZ4 Microscope (Leica Microsystems, Taiwan). For isolates chosen as type cultures of the new species, measurements of all taxonomically useful structures were made and the means and standard errors calculated. We measured morphological features of the asexual structures, these included colony diameter, conidiogenous cells, conidiophores, conidia and denticles (where present). In the case of S. smangaliso we also measured morphological features of the sexual structure (ascomata), we measured ascomata, neck and ascospores. Photographs were taken using a Leica digital camera mounted on the microscope.

1.19.3. Fungal isolation, DNA extraction and sequencing

Fungal isolation, DNA extraction, PCR amplification and sequencing largely followed methods described in Aylward *et al.* (2014a, b) and in this thesis (Ngubane *et al.* 2017 (Chapters 2 and 3)). Ascospores from sporulating ascomata in infructescences were collected using a sterile needle (one isolate per infructescence) and transferred to Petri dishes containing Malt Extract Agar (MEA; Merck, Wadeville, South Africa) emended with Streptomycin Sulfate Salt (0.04 g/L) to minimise bacterial contamination and cyclohexamide (2.5 g/L) to select for members of the Ophiostomatales (Roets *et al.* 2006a). All plates were incubated in the dark at room temperature (*ca*. 21˚C) until purification. A small colony fragment of selected isolates of the suspected new species (Table 4.1) was sub-cultured onto Water Agar (15 g agar/L) and incubated in the dark at room temperature for four days, after which a single hyphal tip was transferred to MEA plates. Plates containing purified cultures were stored in a fridge (10°C) in the dark until further analyses. Additional isolates for one of the undescribed species collected by Roets *et al*. (2009b) were sourced from the culture Collection of Michael Wingfield (CMW), Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa, and included in analyses (Table 4.1). Additional isolates of other taxa needed for analyses in this work are also provided in Table 4.1.

Table 0.1: Isolates of *Sporothrix* taxa collected during fieldwork or requested from the CMW Culture Collection (Pretoria). Data for paratypes from species description literature on various taxa needed for analyses are also shown. All other taxa used in analyses and presented in the tree in the results section (Figure 1) were the same as listed by De Beer *et al.* (2016)

					GenBank		
Species	**Culture**	**Host**	**Collector**	**Location**	**ITS**	**BT**	**CAL**
Sporothrix africana	CMW1812	*P. dracomontana*	MJ Wingfield	Drakensberg, KwaZulu-Natal	DQ316198	Pending	Pending
S. africana	BR1.1	*P. gaguedi*	F Roets	Blyde River Canyon, Mpumalanga Province	Pending	Pending	*
S. africana	BR8.3	*P. gaguedi*	F Roets	Blyde River Canyon, Mpumalanga Province	Pending	Pending	Pending
S. bragantina	CBS430.92	Forest soil	Pfenning & Oberwinkler	Brazil	FN546964	FN547386	*
S. eucalyptigena	CMW44399	*Eucalyptus marginata*	PA Barber	Western Australia, Australia	KU865592	KX273394	KX273427
S. nsini	CMW28602	*P. caffra*	F Roets	Walter Sisulu National Botanical Gardens, Gauteng Province	Pending	Pending	Pending
S. nsini	CMW28603	*P. caffra*	F Roets	Walter Sisulu National Botanical Gardens, Gauteng Province	Pending	Pending	Pending
S. nsini	VM15.1	*P. caffra*	NP Ngubane	Voortrekker Monument, Gauteng Province	Pending	Pending	Pending

S. protearum	VM13.1	*P. caffra*	F Roets	Voortrekker Monument, Gauteng Province	Pending	Pending	Pending
S. smangaliso (*sp. nov. 1*)	DNRD11.3	*P. dracomontana*	NP Ngubane	Royal Natal National Park, KwaZulu-Natal Province	Pending	Pending	Pending
S. smangaliso	BDGP4C1.1	*P. gaguedi*	NP Ngubane	Vryheid, KwaZulu-Natal	Pending	Pending	Pending
S. splendens	GWC9.1	*P. repens*	NP Ngubane	Gouritz, Western Cape Province	*	Pending	Pending
S. thermara	CMW38929	*Euphorbia ingens*	JA van der Linde	Bela-Bela, Limpopo Province	KR051114	KR51102	*
S. zambiensis	CMW29078	*P. caffra*	F Roets	Nchila, Zambia	EU660453	EU660473	Pending

CMW = the Collection of Michael Wingfield, based at the University of Pretoria

CBS = CBS Fungal Biodiversity Centre - an institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

Pending = Sequences have not yet been submitted to GenBank

*Sequences not available

1.19.4. DNA extraction, PCR amplification and sequencing

Fungal mycelia were harvested and placed in Eppendorf tubes with 500 µl TES buffer (100mM Tris-HCl, pH 8.0; 10mM EDTA; 2% (w/v) SDS) with 70 µg PCR grade Proteinase K (Roche Applied Science, Mannheim, Germany) and three glass beads (Möller *et al*. 1992). This solution was thoroughly mixed and cell walls and membranes were disrupted using a Tissue Lyser (Qiagen Retsch, Walpole, MA, USA). Steps described by Möller *et al.* (1992) were followed for DNA extraction and purification.

The Internal Transcribed Spacer (ITS) region was amplified for all isolates using the ITS-1F (5'-CTT GGT CATTT AGA GGA AGT AA-3') and ITS4 primers (5'-TCC TCC GCT ATT GAT ATG C-3') (White *et al*. 1990, Gardes & Bruns 1993). Reaction mixes (25 µl per tube) contained 2.5 µl of 2.5 mM $MgCl_2$, 0.5 µl of each primer, 12 µl 2X KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA), 7.5 µl ddH_2O and 2 µl of 100 ng/µl template DNA. PCR reaction conditions were as follows: an initial denaturation step at 95°C for 2 min, followed by 35 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and delongation at 72°C for 60 s) and termination with a final elongation step at 72°C for 8 min. Further PCR reactions were carried out targeting the Calmodulin (CAL) and Beta-tubulin (BT) gene regions of selected isolates using primers CL3F (5'-CCG ART WCA AGG AGG CST TC-3') and CL3R (5'- TTC TGC ATC ATR AGY TGS AC-3') (Duong *et al.* 2012), and Bt2b (5'-ACC CTC AGT GTA GTG ACC CTT-3') (Glass & Donaldson 1995) and T1 (5'-AAC ATG CGT GAG ATT GTA AGT-3') (O' Donnell & Cigelnik 1977), respectively. Reaction mixtures were the same as those used for the ITS protocol. For samples that failed to amplify, reactions were repeated with 0.5µl DMSO added and/or with a doubling of template DNA concentration. For CAL, the PCR reaction conditions were as follows: initial denaturation step at 95°C for 5 min, followed by 35 cycles (95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 60s), and a final extension step at 72°C for 8 min (Duong *et al.* 2012). The PCR reaction conditions for Beta-tubulin were: an initial denaturation step of 4 min at 94°C, followed by 35 cycles (denaturation at 94°C for 60 s, annealing at 52.5°C for 90 s, and elongation at 72°C for 60 s) and a final elongation step at 72°C for 7 min. All PCR products were sent to CAF (the Sequencing Facility, Central Analytical Facility, Stellenbosch University) for sequencing. Afterwards, base-calling for sequences were confirmed using Chromas V2.6 (Technelysium 1998).

1.19.5. Additional sequences

Using the Basic Local Alignment Search Tool (BLAST) function, ITS sequences were compared to sequences stored on GenBank, the nucleotide database of the National Center for Biotechnology Information (NCBI) (http:// www.ncbi.nlm.nih.gov/blast/Blast.cgi). Blast

searches confirmed that both the putative new taxa belonged to the genus *Sporothrix* (De Beer *et al*. 2016). Closest matches for *Sporothrix sp. nov. 1* included *S. bragantina* (Pfenning & Oberw.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. eucalyptigena* (Barber & Crous) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *S. thermara* (Van der Linde, Six, De Beer, Wingfield & Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf. The closest relatives of *Sporothrix sp. nov. 2* based on these searches included *S. protearum*, *S. splendens* and *S. zambiensis* (*S. splendens* clade). We therefore downloaded data for all three markers for additional taxa based on analyses of all taxa currently described in the genus *Sporothrix* provided by de Beer *et al.* (2016). Additional sequences for *S. bragantina*, *S. eucalyptigena, S. thermara* and *S. zambiensis* were sourced from additional studies (Madrid *et al.* 2010, Roets *et al.* 2010, Osorio *et al.* 2016, van der Linde *et al.* 2016) and all other data were generated in this study (Table 4.1).

1.19.6. Data analyses

Base calling for sequences was verified using Chromas V.2.6 6 (Technelysium Pty Ltd, Tewantin, Australia) and aligned using Bioedit (Hall 1999). For regions in these markers that were difficult to align, further processing was conducted using the Gblocks 0.91b (Castresana 2000) online platform (phylogeny.lirmm.fr) to eliminate areas that were too variable to align. Parameters specified included; 1) setting minimum length of a block after gap cleaning at 5, 2) positions with a gap in less than 50% of the sequences were selected in the final alignment if they were within an appropriate block, 3) all segments with contiguous non-conserved positions bigger than 8 were rejected, 4) minimum number of sequences for a flank position was set at 55%.

Datasets were compiled for the ITS, beta-tubulin and calmodulin markers. These datasets contained 72, 71 and 59 sequences, respectively. After processing using Gblocks the resulting sequences were 663, 807 and 317 base pairs long, respectively. The beta-tubulin sequences sourced using the GenBank numbers in de Beer *et al.* (2016) were all of partial sequences that were approximately 386 base pairs long. Sequences of the species in the *S. splendens* clade were all extended beta-tubulin sequences either sourced from GenBank or sequenced in our lab for this study (Table 0.1). The partial beta-tubulin sequences contained three exons, and two introns; whereas the extended sequences contained four exons and three introns. The exons and introns that were missing from the partial sequences were treated as missing data.

For phylogenetic analyses, Bayesian Inference (BI) and Maximum Likelihood (ML) approaches were used. Prior to BI analyses jModelTest V0.1.1 (Posada 2008) was used to determine the best fitting substitution model using the Akaike information Criteria (Akaike

1974). Analyses for BI were conducted using MrBayes V.3.2 (Ronquist *et al.* 2012) using the model identified using jModelTest. Using the Markov Chain Monte Carlo (MCMC) method, two independent MCMC chains were run simultaneously for ten million generations. Burn-in values, as determined using Tracer 1.4 (Rambaut & Drummond 2007), were used to discard trees. Thereafter, trees were sampled after every 2000 generations. Remaining trees were pooled and the 50% majority rule consensus tree was generated. Missing characters were treated as fifth state characters. RAxML v7.0.4 was used for ML analyses (Stamakis 2014) using the program CIPRES Science Gateway v3.3 (Miller *et al.* 2010). The GTR substitution matrix (determined using jModelTest) and a rapid bootstrap analysis (Stamakis *et al.* 2008) were chosen to search for the best ML phylogeny. In order to determine the branch node confidence, one thousand bootstrap replicates were performed. Trees lower than the burn-in values were discarded. The remaining trees were used to construct a 50% majority rule consensus tree. All analyses were repeated for the three separate data sets and for the three data sets combined.

1.20. Results

For the first undescribed species (hereafter referred to as *Sporothrix smangaliso*) we collected three isolates from two hosts (*Protea dracomontana* and *P. gaguedi*). Collections of *S. smangaliso* were confined to KwaZulu-Natal Province (Vryheid and Drakensberg National Park). For the second new species (hereafter referred to as *Sporothrix nsini*) we collected 23 isolates from three hosts (*P. caffra, P. dracomontana* and *P. gaguedi*) and obtained two isolates (*P. caffra*) from previous collections (Roets *et al.* 2006). Multiple individuals of this species were collected per population: seven from the Drakensberg (three from *P. dracomontana* and four from *P. caffra)*, four from the Voortrekker Monument (*P. caffra*), seven from Faerie Glen (*P. caffra*), two from Rustenburg (*P. caffra*) and three from Blyde River Canyon (*P. gaguedi*).

The tree obtained from Baysian analyses of the combined data set is provided in Figure 4.1 (statistical support and parameters in Table 4.2). As the topology of the tree obtained from ML analyses was largely congruent with the tree obtained from Bayesian analyses, bootstrap values are also indicated on this tree. Analyses of the separate markers always placed *Sporothrix nsini* in a clade with other *Protea*-associated taxa such as *S. africana*, *S. protearum* and *S. zambiensis* with strong support (data not shown). Support for its placement as sister to these taxa were, however, only strong when analysing the ITS, beta-tubulin, and calmodulin combined data sets due to lack of informative data in the Gblocks treated data sets (data not shown).

Analyses of the separate markers did not always place *S. smangaliso* in the same position as when analysing the combined data set. For example, when the beta-tubulin data was analysed using BI it was placed with all the taxa in groups G and H of De Beer *et al*. (2016) with an unresolved placement of all the species within (posterior probability= 0.95), but when analysed using ML it was placed within clade G (ML bootstrap= 19%). Analyses of the ITS dataset placed this taxon in group G as sister to *S. curviconia* de Hoog (ML bootstrap= 53%). Analyses of the calmodulin dataset, on the other hand, placed this taxon in clade H as sister to *S. bragantina* (ML: bootstrap= 85%, and BI: posterior probability= 0.98). On the combined tree, however, *S. smangaliso* was placed in group G as sister to *S. curviconia* (ML: bootstrap = 98%, BI: posterior probabilities = 1). Molecular data alone therefore cannot congruently place this taxon to its closest taxon, but it does place it within clades including species such as *S. bragantina* and *S. curviconia*.

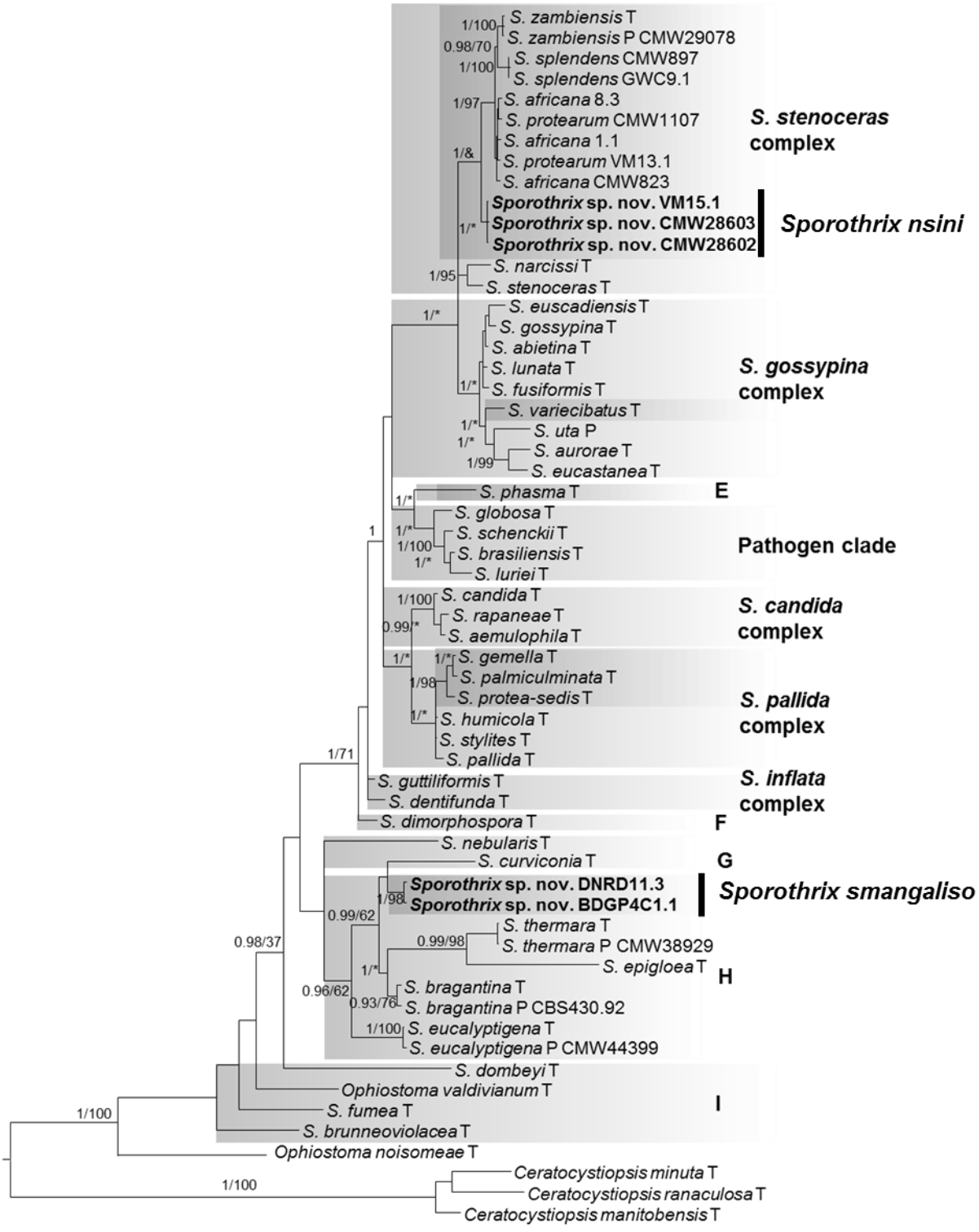

Figure 0.1: Combined tree (CAL, ITS and BT) for *Sporothrix* including the two new species. Taxa highlighted in dark grey are *Protea*-associated, with taxa in bold representing isolates of the new taxa described here. Support values on the left indicate posterior probabilities (significant at >0.95, Bayesian inference) and the values on the right are bootstrap values (significant at >70, ML). Nodes marked with an asterix (*) denote nodes without support or that do not exist on the ML based topology. Isolates not followed by isolate numbers and names of complexes in the tree were those listed in De Beer *et al.* (2016). Data for taxa that are followed by isolate numbers were either obtained from previous studies (Madrid *et al.* 2010, Roets *et al.* 2010, Osorio *et al.* 2016, Van der Linde *et al*. 2016) or were generated in this study (Table 4.1). T = holotype, P = paratype.

Table 0.2: Parameters used and statistical values yielded from maximum likelihood (ML) and Bayesian inference (BI) analyses of the four datasets (combined, ITS, beta-tubulin and calmodulin)

	Dataset		Combined	ITS	BT	Cal
ML	Substitution model		GTR+I+G	GTR+I+G	GTR+I+G	GTR+I+G
	Gamma shape		0.23	0.23	0.30	0.20
	Log-likelihood		-8158.43	-2564.83	-3699.73	-2244.78
	Relative rate parameters	r(A<->C)	1.83	2.03	1.19	2.73
		r(A<->G)	6.29	1.80	6.08	18.91
		r(A<->T)	2.59	2.53	2.02	3.47
		r(C<->G)	0.88	0.60	1.31	1.77
		r(C<->T)	4.50	4.54	5.07	5.33
		r(G<->T)	1	1	1	1
	Nucleotide frequencies	pi(A)	0.22	0.22	0.24	0.19
		pi(C)	0.28	0.33	0.24	0.27
		pi(G)	0.30	0.27	0.32	0.32
		pi(T)	0.20	0.19	0.19	0.23
BI	GTR submodel probability		0.03	0.05	0.07	0.10
	Model string		121313	111123	123323	121313
	Gamma shape		1.00	0.34	0.31	0.19
	Relative rate parameters	r(A<->C)	0.08	0.14	0.08	0.08
		r(A<->G)	0.34	0.19	0.34	0.64
		r(A<->T)	0.09	0.17	0.09	0.08
		r(C<->G)	0.09	0.19	0.09	0.06
		r(C<->T)	0.33	0.31	0.33	0.10
		r(G<->T)	0.08	0.07	0.08	0.03
	Nucleotide frequencies	pi(A)	0.26	0.17	0.26	0.18
		pi(C)	0.22	0.36	0.22	0.26
		pi(G)	0.31	0.28	0.31	0.31
		pi(T)	0.21	0.19	0.21	0.25

Based on phylogenetic analyses the newly collected taxa are recognised as distinct and undescribed taxa in the genus *Sporothrix*. These are described here as *S. smangaliso sp. nov*. and *S. nsini sp. nov*.

1.20.1. Taxonomy

Sporothrix smangaliso N.P. Ngubane, L.L. Dreyer, K.C. Oberlander and F. Roets *sp. nov.* (Figure 4.2 A–D)

Etymology: The epithet *smangaliso* is derived from isiZulu meaning surprise or miracle, and refers to the unexpected discovery of this fungus that is very different from all the other *Protea*-associated *Sporothrix* species and phylogenetically distantly related to them.

Only a single ascomata of this taxon could be traced within infructescences from initial collections. We therefore provide measurements for the sexual stage of this fungus based on a single ascomata and the ascospores it contained. Ascomata superficial on the host substrate. Base globose, 110 µm in diameter, dark brown to black, with hyphal ornamentation. Neck black, 71.7 µm long without osteolar hyphae. Asci evanescent. Ascospores reniform, hyaline, sheaths absent, 2.05–3.61 (2.95±0.11) µm long and 0.99–1.83 (1.45±0.07) wide. Colonies white to cream coloured on MEA, odourless, circular with entire edge and woolly surface. Colonies reach 16 mm after 6 days when grown on MEA at 24°C. Conidiophores are hyaline. Conidiogenous cells 2.6-21.9 µm, cylindrical, arising directly from hyphae, hyaline, becoming denticulate. Denticles up to 0.5 µm long. Conidia hyaline, aseptate, guttuliform or fusiform, 3.62–6.60 (4.69±0.3) µm long and 1.65–2.50 (2.02±0.1) µm wide, arising in bunches from conidiogenous cells or singly from hyphae.

Substrate: Isolated from decaying florets within the infructescences of *P. dracomontana* from Royal Natal Nature Reserve (Drakensberg), and from *P. gaguedi* from Bivane Dam (Vryheid), KwaZulu-Natal Province.

Distribution: KwaZulu-Natal Province (Drakensburg to Vryheid).

Specimens examined: South Africa, KwaZulu-Natal Province, Bivane Dam (Vryheid). Isolated from *P. gaguedi* infructescences, May 2015. NP Ngubane, (PREM Pending, CMW Pending, CBS Pending, Holotype) and (PREM Pending, CMW Pending, CBS Pending, Paratype); South Africa, KwaZulu-Natal Province, Royal Natal Nature Reserve (Drakensberg), *Protea dracomontana* May 2015, NP Ngubane (PREM pending, CMW pending, CBS pending, Paratype).

Notes: Based on the morphology of the teleomorph, *S. smangaliso* is distinctly different from the rest of the species in group H. The ascomata of *S. smangaliso* appear to be much smaller than that of *S. bragantina* (ascomata: (130–) 220 µm X 700 (–1200) µm) (Pfenning & Oberwinkler 1993), although only a single ascomata could be found when revisiting collected material. Morphologically these species are also most similar. The ascopores of both species are the same size and are reniform in shape (Pfenning & Oberwinkler 1993). Their conidia are also both hyaline, guttuliform or fusiform, pointed at the base, smooth-walled and the same size (Pfenning & Oberwinkler 1993). Both species form prominent denticles on conidiogenous cells. The perithecial neck of *S. smangaliso* is, however, the shortest in the

group (*S. eucalyptigena* (100 µm–500 µm) (Barber and Crous 2015) and *S. bragantina* (700–1200 µm)) (Pfenning & Oberwinkler 1993). Based on BLAST searches using the calmodulin marker, *S. smangaliso* isolates group closest to isolates of *S. bragantina* (*S. mangaliso* isolate BDGP2C1.1 had 75% coverage with 90% identity and 2% gaps with *S. bragantina* isolate CBS 474.91 (Genbank: KX590784.1). The ITS sequence of *S. smangaliso* isolate BDGP4C1.1 had 91% coverage with 97% identity and no gaps with *S. bragantina* isolate CBS 474.91 (Genbank: NR137153.1). Similarly, the beta-tubulin sequence of *S. smangaliso* isolate BDGP4C1.1 was the closest to the *S. bragantina* isolate CBS 474.91 (Genbank: FN547387.1) with 37% coverage, 87% identity and 4% gaps.

Sporothrix nsini N.P. Ngubane, L.L. Dreyer, K.C. Oberlander and F. Roets *sp. nov.* (Figure 4.2 E–H).

Etymology: The epithet *nsini* is derived from isiZulu meaning toothless, and refers to the absence of denticles on the conidiogenous cells of this species.

Ascomata not seen. Colonies cream coloured on MEA, odourless, circular with entire edge and smooth, glossy surface. Colonies reach 12 mm after 6 days when grown on MEA at 24°C. Conidiophores hyaline. Conidiogenous cells 1.1–55.3 µm, cylindrical, arising directly from hyphae, hyaline, not denticulate. Conidia hyaline, aseptate, obovate, 3.43–6.59 (4.4±0.1) µm long and 1.92–3.60 (2.54±0.1) µm wide, arising directly from conidiogenous cells or very often only from tips of conidiogenus cells.

Substrate: Isolated from decaying florets within the infructescences of *P. caffra* from Faerie Glen, Royal Natal Nature Reserve (Drakensberg), Rustenburg and Voortrekker Monument; from *P. dracomontana* from the Royal Natal Nature Reserve (Drakensberg), and from *P. gaguedi* from the Blyde River Canyon.

Distribution: from KwaZulu-Natal Province (Drakensburg) to Mpumalanga Province (Blyde River Canyon), South Africa.

Specimens examined: South Africa, Gauteng Province, Walter Sisulu National Botanical Gardens. Isolated from *P. caffra* infructescences, April 2005. F. Roets. (PREM Pending, CMW Pending, CBS Pending, Holotype) and (PREM Pending, CMW Pending, CBS Pending, Holotype); South Africa, Gauteng Province, Voortrekker Monument, *P. caffra* infructescences, May 2015, N.P. Ngubane (PREM pending, CMW pending, CBS pending, Paratype).

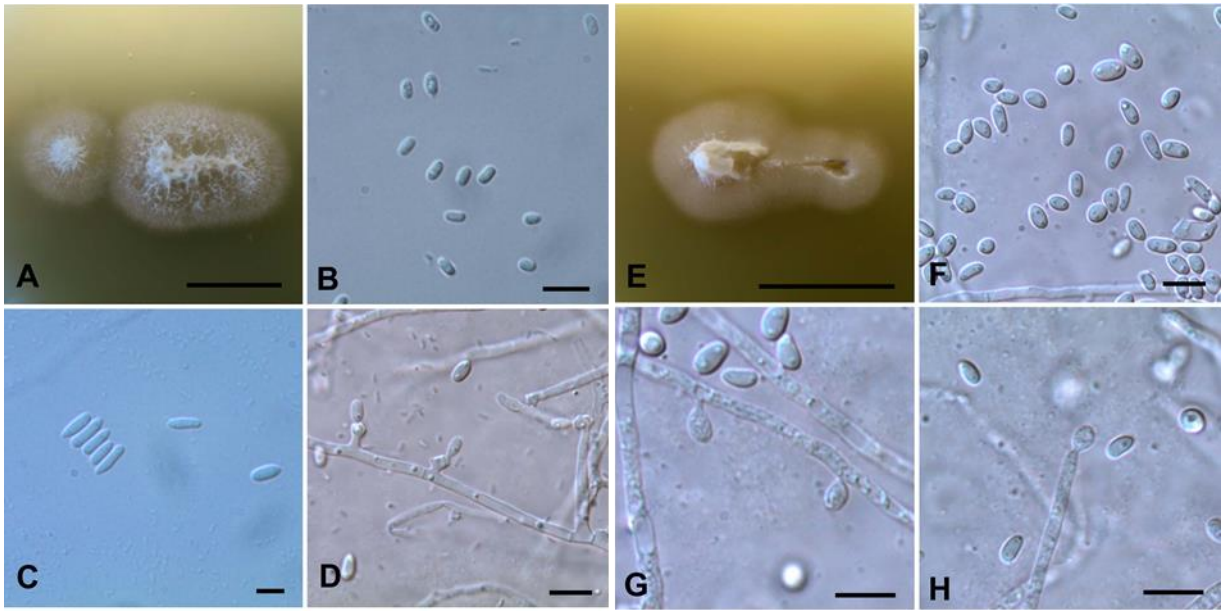

Figure 0.2: Micrographs of *Sporothrix smangaliso* (A-D) and *S. nsini* (E-H). A. Colony on Malt extract agar. B. Ascospores. C. Conidia. D. Conidiogenous cells with budding conidia and prominent denticles. E. Colony on Malt extract agar. F. Conidia. G. Conidia arising directly from hyphae. H. Typical formation of conidia at tip of conidiogenous cell (note the absence of denticles). Scale bars: A and E = 10 mm, the rest = 5 µm.

1.21. Discussion

The discovery of these two *Protea*-associated *Sporothrix* species brings the total number of *Sporothrix* species on *Protea* to 11. Of the 11 groups and complexes demarcated in de Beer *et al.* (2016), five contain at least one *Protea*-associated *Sporothrix* species. The discovery of a species belonging in group G or group H (delineated by de Beer *et al.* (2016)) has added support for multiple origins of *Sporothrix* in this unusual niche (Roets *et al.* 2006a). Reasons for the high ecological suitability of this niche by varied *Sporothrix* species remains uncertain, but may be linked to the abundance of nutrient sources from decaying floral parts (Aylward 2017), high moisture availability (Roets *et al.* 2012) and abundance of potential vectors in the form of arthropods (Roets *et al.* 2006b, 2007, 2009a). However, competition for resources (space, nutrients and vectors) is expected to be very high between the different taxa as *Protea* infructescences are relatively small. The numerical dominance of taxa such as *S. protearum* and *S. africana* on the same hosts as the newly described species is intriguing and suggests that they are much better competitors in this system.

Morphologically, *Sporothrix nsini* closely resemble other *Protea*-associated species in the *S. stenoceras* complex. Its colony morphology is very similar to that of the other *Protea*-associated *Sporothrix* species in the *S. splendens* clade. Its conidia are longer than those of *S. africana* (1.3 µm). Other than this, the morphological measurements were similar to those of all the other species in this clade. The most notable morphological feature that distinguishes *S. nsini* from all other species in this clade is the lack of denticles on the

conidiogenous cells. Speciation after initial colonisation onto *Protea* has occurred in at least two clades, the *S. splendens* clade and the *S. pulmiculminata* clade (Roets *et al.* 2006a, 2010, de Beer *et al.* 2016). The relationship of *S. nsini* as sister to all other known taxa in this clade from *Protea* presents additional questions regarding the evolution of these taxa. It occurs on numerous hosts (the same hosts of *S. africana* and *S. protearum*) and does not numerically dominate either of these (Ngubane *et al.* 2017 (Chapter 2)). It is also known from numerous localities, and in most instances the same localities as *S. africana* (Ngubane *et al.* 2017 (Chapter 2)). This species is also known to grow in sympatry with at least *S. africana* (within the same *P. caffra* infructescence at Voortrekker Monument). Reasons for speciation in this group of species are therefore not clearly related to host relationships or geographic locality (as may be the case for other taxa such as *S. zambiensis* and/or *S. splendens*), but may include combinations of these factors. It is also possible that the different taxa are preferentially associated with different vectors, such as different mite or beetle species in this niche (Roets *et al.* 2006a, 2009a, 2010). However, as was shown for similar fungi from the *Protea*-associated niche in the Cape Floristic Region of South Africa, these associations are not always clear as host chemistry and vector identity only partly explain associations (Roets *et al*. 2012). Future studies should investigate the host and vector associations of species in this complex in a competitive-interactions arena.

Depending on the molecular marker used, *S. smangaliso* belongs to either group G or H. These groups currently contain the species *S. curviconia*, *S. nebularis* (Romón, de Beer, Zhou, Duong, Wingfield, Wingfield) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *S. nigrograna* (Masuya) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. bragantina*, *S. thermara*, *S. eucalyptigena* and *S. epigloeum* (Guerrero) Z.W. de Beer, T.A. Duong & M.J. Wingf., respectively (de Beer *et al.* 2016). However, based on the calmodulin marker that is recommended for discerning between closely related Ophiostomatales (de Beer *et al.* 2016), S. *smangaliso* most likely belongs in Group H as sister to *S bragantina*. Similarly, based on closest matches using BLAST searches and full length sequences (*i.e*. not Gblocks treated data as in our phylogenetic analyses), *S. smangaliso* is most closely related to *S. bragantina* rather than *S. curvicona* as suggested by our analyses of the combined data set. The morphology of these taxa also closely match. Species Group G and Group H seem to have interesting phylogenetic relationships as the members within these groups move freely between the two groups depending on the marker used (de Beer *et al.* 2016). *Sporothrix curviconia* (Clade G) is an associate of *Terminalia ivorensis* huh A. Chev. in the Ivory Coast (de Beer *et al*. 2016), but also moves to group H containing *S. bragantina,* a Brazilian soil associate (Pfenning *et al.* 1993), *S. eucalyptigena* an associate of *Eucalyptus marginata* Donn ex Sm. in Australia (Barber and Crous 2015) and *Barringtonia racemosa* (L.) Spreng.

in South Africa (Osorio *et al.* 2016), and *S. thermara,* which is an associate of a native *Euphorbia ingens* E.Mey. ex Boiss. in South Africa (van der Linde *et al.* 2016) depending on the specific marker used. The *S. bragantina* clade (group H) is therefore an interesting and diverse assemblage of taxa without any clearly defined ecology. The collection of *S. smangaliso* here may therefore represent chance events of movement of this fungus from a different habitat and indicate that it may not be specifically associated with this niche. However, another undescribed *Sporothrix* species (discovered through environmental sequencing) that is similar to *S. smangaliso* based on ITS data is known from the infructescences of *Protea repens* in a very different environment from where *S. smangaliso* was collected (environmental sequencing; Zander Human, *pers*. *com*.). It was isolated from the infructescences of this *Protea* species in a unique biome, the Fynbos, that bares little similarity to the grassland or savannah biomes where *S. smangaliso* is known from. Given the substantial distance between these localities (more than 1000 km) and the different host species, these two *Sporothrix* species may be representative of a third lineage in which speciation has occurred in this niche. However, more collections from more hosts and additional localities are needed to verify this.

The discovery of 11 species from the spatially limited *Protea* niche belonging to five distinct clades suggests that the association between *Protea* species and *Sporothrix* species may be more complex than initially thought. Sampling from additional localities and in greater depth (via environmental sequencing) is required to fully appreciate the diversity and ecologies of *Protea*-associated *Sporothrix* species. Sampling of other habitats for *Sporothrix* should also intensify in order to clarify the full ecological range of *Protea*-associated species. Coupled to this, studies should also focus on vector relationships of all species to elucidate the factors that maintain species barriers, especially in closely related taxa such as those in the *S. splendens* clade. To conclude, it is clear that the infructescence niche represents a very complex micro-ecosystem with numerous trophic levels and lends itself to the study of cascading effects of ecosystem interferences.

1.22. References

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GENERAL CONCLUSIONS

Since the discovery of the first *Protea*-associated ophiostomatoid fungus, *K. proteae* (then thought to belong to the genus *Ceratocystiopsis* (Wingfield *et al.* 1988)), the body of knowledge on their diversity and general biology has been steadily accumulating. To date, three *Knoxdaviesia* (de Beer *et al.* 2013) and nine *Sporothrix* species have been described and some of their vectors identified (Roets *et al*. 2007, 2009, 2013). Recently, the investigations into the factors that govern host selection and levels of host exclusivity have commenced (Roets *et al.* 2012). While the exact factors that dictate host selection and exclusivity are not known, factors that partially influence them have been identified as host chemistry and temperature (Roets *et al.* 2012). The nature of the relationship between these fungi, their hosts and vectors are therefore very complex. In 2014, the study investigating the population structure of the *Protea repens*-associated *Knoxdaviesia proteae* pioneered research into the micro-ecology of these enigmatic fungi in attempt to better understanding the relationship between these fungi and their vectors (Aylward *et al.* 2014, 2015). Not only did this study raise additional questions about the dispersal ecology of *Protea*-associated ophiostomatoid fungi but it also highlighted the gap in the knowledge about the population dynamics of not just the *Knoxdaviesia* species but also that of *Protea*-associated *Sporothrix* species. This gave rise to the main aim of the thesis presented here in which the population genetics of *Sporothrix* species associated with *Protea* species within and outside the Core Cape Subregion (CCR) was studied.

Population genetic analysis of *Sporothrix splendens* showed that the populations of *Protea*-associated ophiostomatoid fungi within the CCR are strongly influenced by long distance dispersal of their propagules thus giving rise to populations with nearly no geographic structure. This was very similar to what was found for *K. proteae* in previous studies (Aylward *et al.* 2015). The vastness of distances between populations investigated highlight the need for research into additional secondary vectors since the beetles implicated as secondary vectors are unlikely to cover these distances especially not frequently enough for such weak signalling for geographic structure to emerge. Aylward *et al.* (2015) proposed that birds (which are known to carry mites (Proctor & Owens 2000)) are very likely involved in the dispersal of these ophiostomatoid fungi, at least, at a secondary vector capacity. Along with previous studies on these intriguing fungi (Aylward *et al.* 2014, 2015, 2017), the present study proposes a central role of birds in the dispersal of *Protea* flower-associated fungi, a process that has not yet been clarified.

The population structures of *S. africana* and *S. protearum* suggest that these two taxa represent a single species, *S. protearum*, with a similar population structure as that of *K.*

capensis found in the CCR on multiple hosts (Aylward *et al.* 2017). The genetic diversity in this species is remarkably high, similar to that observed in all the ophiostomatoid fungi studied so far in *Protea*. Similar to their Cape counterparts, gene flow was very high and played an important role in eliminating the signal for isolation by distance or genetic isolation based on host identity. Evidence of complete sympatry and phylogenetic relatedness between *S. africana* and *S. protearum* supports the notion that these taxa should be one species. It is thus curious that *S. nsini sp. nov*, sister to species in the *S. splendens* complex and that grows in sympatry with *S. africana* and *S. protearum,* is by all accounts clearly a distinct separate species. This highlights the need to better understand the complexity of the relationship between these fungi and their hosts and vectors, and how species boundaries are maintained. The discovery of *S. smangaliso* that is phylogenetically quite distinct from other *Sporothrix* species known from *Protea*, adds another level of mystery surrounding these enigmatic fungi. This fungus is closely related to taxa found in association with bark beetles and conifers, soil and other niches (de Hoog 1974, Barber & Crous 2015), yet its biology probably bares very little resemblance to their biology and is rather very similar to that of other *Protea*-associated *Sporothrix* species. Its discovery in sympatry with *S. africana*, *S. nsini sp. nov.* and *S. protearum* provides some evidence for shared biologies. Given the low numbers of this fungus collected it is likely that it is mainly associated with other ecological niches. As its closest relative seems to be *S. bragantina*, it is a strong possibility that *S. smangaliso* may be a soil-associated species.

The description of these two new species brings the total of *Sporothrix* species known from *Protea* to 11. Seven of these are found outside the CCR within *Protea caffra*, *P. dracomontana* and *P. gaguedi*. Out of the 11 clades identified in de Beer *et al.* (2016), five contain a *Protea*-associated *Sporothrix* species supporting the proposal made by Roets *et al.* (2013) that there have been multiple events of invasion into the *Protea* niche followed by species radiations in some clades. The thesis presented here indicates that reasons for speciation are only partly explained by geography and host relationships and further investigations, including population genetic analyses of all taxa and intensive studies on vectors, are needed. The occurrence of many taxa in sympatry also raises questions regarding competitive abilities of the fungi and should form the basis of future interaction studies. These studies should ideally include investigations into differences in substrate use of the different taxa as this may partly explain host relationships and clarify whether all taxa are indeed saprobes as speculated.

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